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The Relationship between Hormone-induced Tissue Growth and Neoplasia: *A Review*

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This review will present a general appraisal of existing information rather than a detailed or comprehensive review of the subject to be discussed.

The terms to be employed require some definition. "Hormone-induced tissue growth" refers to an increment in tissue mass resulting either directly or indirectly from the action of exogenous or endogenous hormones or hormone-like substances. Such increments in tissue mass may involve one or several end organs, or, as in the case of the growth hormone, all the tissues of the body may be affected. The increased tissue mass may represent actual cellular proliferation or increased cell size. Moreover, the increased mass may in some instances be contributed to by secretions, interstitial fluid, ground substance, or even increased vascularity of the hormone-sensitive organ. Thus, the term "growth" in this discussion is employed in a broad sense.

"Neoplasia" is the formation of a tissue not regularly encountered in organisms of a given generic group. Accordingly, the term neoplasia may be properly employed only when the observer has an adequate knowledge of the regularly encountered variations seen in the tissues of the particular form under study.

In keeping with these definitions it is clear that hormone-induced tissue growth will have in some instances certain features which set it apart from neoplasia, and in other instances the differences may not be very decisive.

In this discussion it will be our aim to review the interrelationship between these two forms of new tissue formation in the light of the more significant observations in this field.

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The most intensively studied forms of hormone-induced tissue growth are those brought about by the action of the "trophic" factors of the anterior pituitary gland and the gonadal and adrenal steroids.

Smith's (53) classical study on the effects of hypophysectomy in the rat established the dependence of the entire body growth upon the normal secretion of pituitary growth hormone or "somatotropin" (17). Recent studies of Moon *et al.* (39–41) describe the highly frequent occurrence of neoplasms in rats treated with "growth hormone" for prolonged periods of time. It is noteworthy that these neoplasms are of widely different types and include sarcomas, lymphoid tumors, and adenocarcinomas. Attention should also be called to the fact that the pituitary growth hormone is now widely considered to be identical with the so-called "diabetogenic factor" of the hypophysis (9, 10, 31). Some evidence is at hand indicating that patients with diabetes have a higher incidence of cancer than comparable population groups (33). In the case of endometrial carcinoma, frequent association with diabetes has been convincingly reported by several independent observers (4, 26, 42). Thus, the observations of Moon *et al.* may have some bearing on the clinical association of diabetes and cancer, although these studies do not include information on the carbohydrate tolerance of growth hormone-treated rats. Nevertheless, one is led to consider that the tissue-growth effects of pituitary somatotropin, mediated as they are through measurable effects upon carbohydrate and protein metabolism, probably overlap in some way with the tumorigenic effect of the hormone.

The thyreotropic or thyroid-stimulating hormone (TSH) of the anterior pituitary not only sup-

ports normal thyroid tissue growth and metabolism but has been shown to affect neoplastic thyroid tissue as well (50, 54). Cases of thyroid carcinoma presenting clinical manifestations of thyreotoxicosis indicate that TSH may play a definite role in the pathogenesis of thyroid malignancy. Experimental derangement of thyroid-pituitary relationships by prolonged thiouracil administration in mice has led to the formation of ectopic masses of noninvasive thyroid tissue in the mouse and localized thyroid tumors in the rat, but these are not considered true malignancies (13, 38). However, Purves and Greisbach (49) interpreted the thyroid tumors found after 20 months of ingestion of thiourea as malignant, their criteria for malignancy being intravascular extension and pulmonary metastases. It has also been shown that thiouracil accelerates and augments the formation of thyroid carcinomas induced by 2-acetylaminofluorene, suggesting some interaction between the thyreotropic and the carcinogenic response (7, 47).

Experience with the therapeutic use of radioactive iodine in thyroid carcinoma indicates that at least some thyroid malignancies possess specific affinity for iodine, a function which, in turn, is TSH-dependent. The way in which TSH may affect the genesis and course of thyroid cancer is still obscure, but sufficient data are at hand to indicate that there are some common hormonal factors upon which both normal and malignant thyroid tissue are highly dependent.

Cushing originally attributed the pathogenesis of the clinical syndrome which now bears his name to abnormal hormone production from basophile adenomas of the anterior pituitary. Cushing's disease is now generally considered to be a manifestation of hyper-adrenocorticism arising from adrenal tumor or hyperplasia and associated with morphological and functional pituitary abnormalities (2, 11). Thus, on clinical grounds there exists rather cogent evidence that adrenal neoplasms represent some derangement in the normal trophic influences exerted by the anterior pituitary upon the adrenal. This approach is strengthened by the experimental production of adrenocortical carcinomas in mice following gonadectomy very early in life, so as to produce a prolonged period of pituitary-adrenal imbalance (58). The prevention of such adrenal tumors by the administration of various pituitary-depressant steroids convinces one that the constant play of qualitatively or quantitatively abnormal adrenotrophic influences upon the adrenal cortex is essential for the development of neoplasia in this hormone-sensitive tissue (59).

Abundant experimental evidence indicates that gonadal tumors may result from a derangement in the normal pituitary-gonadal relationship. The development of malignant tumors in the intrasplenically placed ovary is a phenomenon of great significance (8, 34, 37). These ovarian tumors apparently result from the prolonged and excessive bombardment of the grafted ovary by the unrestrained gonadotropic activity of the anterior pituitary. The initial response to this type of pituitary stimulation is a normal type of hormone-induced tissue growth. Subsequently, a progressive change to malignant degeneration may be discerned. The induction of testicular tumors by intrasplenic transplantation (55) or by prolonged estrogen administration (28) similarly emphasizes the critical significance of an altered pituitary-gonadal relationship in the pathogenesis of such tumors.

In marked contrast to the thyroid, adrenals, and gonads, the mammary gland represents a pituitary-sensitive organ in which frequently occurring malignancies have thus far not been convincingly associated with a demonstrated abnormality of mammotropic pituitary function (51). However, the critical role of steroid factors in the pathogenesis of mammary tumors has been amply demonstrated both clinically and experimentally (6, 14). Furthermore, the demonstrated therapeutic value of massive estrogen and androgen administration in clinical breast cancer (25, 44, 57) serves to emphasize the dependence of such tumors upon an optimal steroid balance. Nevertheless, none of these data show any decisive relationship between the normal trophic response of mammary tissue to steroidal hormones and true mammary neoplasia. The significant observations of Gardner (19) and of Foulds (18) in certain strains of mice in which proved mammary carcinoma regresses following parturition indicate a partial dependency of such breast tumors on the hormonal factors of pregnancy. Clinical observation of the exacerbation of mammary carcinoma by pregnancy supplements the experimental data in this regard (52).

Greene (23) has described a strain of rabbits, presenting evidence of hyperestrinism, in whose mammary glands progressive changes from the proliferative to the neoplastic state could be clearly demonstrated. The induction of mammary carcinoma in estrogenized male mice (51) simply indicates that an infantile gland cannot be the site of a malignancy. This is borne out in clinical observation, since to the writer's knowledge there has not been recorded a single instance of prepuberal breast cancer in the human female.

The occurrence of malignant tissue in the breasts of human males following prolonged estro-

gen administration for prostatic carcinoma is highly suggestive of a carcinogenic action of the administered estrogen (1, 21, 35). Recent personal experience with a previously untreated patient who presented simultaneous breast and prostatic cancer suggests the possibility of a frequent spontaneous association of these two malignancies somewhat paralleling the frequent association of cervical and breast cancer in the human female. Twombly has recently reported (56) the histochemical differentiation between a prostatic metastasis to the breast and primary breast malignancy by the finding of a high acid phosphatase content in the tissue under study. Accordingly, some degree of reservation is justified in concluding that estrogens have been shown to induce mammary carcinoma in the human male.

In the secondary sex organs of the male and female genital tract the relationship of the trophic action of steroidal hormones to the genesis and cause of neoplasia is obscured by a very large body of conflicting experimental and clinical observation. The dramatic regression of prostatic carcinoma following orchiectomy or following the anti-androgenic action of estrogens reflects in a striking way the marked dependency of malignant as well as normal prostatic tissue upon endogenous androgen (32). One should not conclude, however, that endogenous androgen is directly involved in the pathogenesis of prostatic carcinoma. The widely discussed but poorly documented phenomenon of the absence of prostatic carcinoma in eunuchs would favor such an inference. However, we lack any experimental or clinical demonstrations of the induction of prostatic carcinoma by androgen administration. In fact, Horning's observations suggest that reactivity to androgen on the part of the prostatic epithelial cell of the mouse suppresses the carcinogenic response to administered methylcholanthrene (29, 30). In our hands[1] the administration of massive doses of androgen for as long as 18 months to the adult mongrel dog yielded neither metaplastic nor neoplastic change in the prostate. Since both the dog (43) and the monkey (15) are subject to spontaneous carcinoma of the prostate, further experimental work on the actual role of androgen in the genesis of prostatic carcinoma is indicated.

The relationship between estrogen-induced tissue proliferation and neoplasia in the female genital tract is of great practical and theoretical importance. The numerous animal studies afford only mixed information. Lipschütz (36) has recently reviewed the studies from his laboratory concerning the "tumorigenic" action of the steroids in the guinea pig. These experiments have demonstrated that estrogenic steroids can induce fibroid tumors. It is striking that not once in these prolonged and extensive studies did the authors encounter a truly malignant, invasive, and metastasizing tumor in the guinea pig. In direct contrast are the reports of Gardner (20) concerning the occurrence of highly malignant cervical carcinomas in mice following prolonged estrogen administration. Similarly, in the strain of rabbits described by Greene (23), the association of hyperestrinism with spontaneous carcinoma of the cervix is highly suggestive of a causal relationship. However, the failure to produce experimentally malignancy of either the breast, cervix, or endometrium in the *Macacus rhesus* after a decade of continuous estrogen administration leads one to hesitate in generalizing from one species to another (16, 48). Similarly, Crossen and Suntzeff (12) report the occurrence of polyposis and hyperplasia but no malignancy in the endometrium of an aged monkey treated for a prolonged period with estrogen and progesterone. This failure of malignancy in the estrogen-treated primate is rendered even more significant when one considers the marked metaplastic changes readily produced in the cervical glands of the monkey by estrogen administration (27, 45).

From the foregoing, it is clear that only clinical evidence may be regarded as directly pertinent to the critical problem of the potential carcinogenicity of estrogens in the human female. A number of scattered clinical case reports has offered some suggestion of a causal relationship between estrogen administration and clinically validated cases of carcinoma of the breast and uterus (3, 5, 22, 46). The evidence presented by these reports must be regarded as largely inferential, being based upon the "*post hoc, propter hoc*" principle. In contrast to these isolated reports, the systematic observation of the effects of massive and prolonged estrogen administration in patients with breast cancer has not revealed the induction of genital tract carcinoma in an extensive series of cases treated continuously for from 3 months to 2 years.[2] This report is all the more significant, since it deals with a group of breast cancer patients who on statistical grounds may be expected to be more prone to genital tract carcinoma than other females of comparable age.

On the other hand, the histopathological studies of Gusberg and of Hertig and Sommers (24, 26) lead one to consider very carefully the potential role of both endogenous and exogenous estrogen in

[1] R. Hertz and W. W. Tullner, unpublished data.

[2] Walton Van Winkle, Jr. Committee on Therapeutic Trials of A.M.A. (personal communication).

the genesis of uterine cancer. The frequent associations of fundal cancer with anatomically demonstrable ovarian lesions capable of quantitatively and qualitatively abnormal steroid production suggests either a causal relationship between the two lesions or a common cause for both. Insufficient biochemical data are at hand in the actual cases described by these authors to warrant a final conclusion on this point. In fact, the multiple associations of fundal cancer with obesity, hypertension, and diabetes, as well as with the ovarian lesions, suggest some common etiologic factor for all these endocrine manifestations (4, 42, 26).

In the light of these conflicting and indecisive data, the writer desists from any definitive conclusion regarding the carcinogenicity of estrogens in the human female. From a practical point of view, it would seem appropriate (*a*) to restrict the clinical use of estrogen to only those patients who present a clear indication for its use, (*b*) to take all available steps to eliminate the possible pre-existence of carcinoma in the breasts or pelvis of any patient to be given estrogens, and (*c*) to follow carefully all estrogenized patients by frequent physical examination and cytological study of the vaginal smear. Under these conditions, available evidence would indicate that prolonged estrogenization is a reasonably safe form of therapy. From the biological point of view, the bulk of the evidence would indicate to the writer that in the human subject estrogens exert a feeble carcinogenic action, if any.

It is thus clear that a large gap exists in our knowledge of the relationship between hormone-induced tissue growth and the neoplastic process. It is equally clear that these two processes overlap sufficiently to demand the closest scrutiny of their possible interrelationships. Thus, the experimental production of malignancy by simple hormonal imbalance (8, 28, 58) challenges the clinician to review the earlier medical history of patients with comparable malignancies in order to ascertain whether at any time they may have presented a picture of endocrine imbalance. In this way, the complete characterization of a premalignant state may become clear. Further, these manifestations of active humoral interrelationships serve to support the view that neoplasia is a local manifestation of a metabolic disorder. Thus, diagnostic and therapeutic efforts must be directed systemically as well as locally.

It may be argued that this approach is to be applied only to malignancies in the hormonally active organs discussed above. Rather, one should consider that such profound hormonal interactions probably exist among all the various tissues of the body, but endocrinology has thus far only uncovered the more obvious humoral relationships.

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Study of Liver Tumor Development and Histologic Changes in Other Organs in Rats Fed Azo Dye 3′-Methyl-4-Dimethylaminoazobenzene*

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Pathological changes induced in the liver of rats sustained on a diet containing 3′-methyl-4-dimethylaminoazobenzene (*m*′MeDAB) have not been extensively reported. Early liver changes, following administration of the dye for 12 weeks, were studied by Cunningham, Griffin, and Luck (2). Studies on the effect of this carcinogen with variations in diet were made by Griffin, Clayton, and Baumann (5). Our experiments with *m*′MeDAB were made to study cell changes during the development of liver tumors and to determine the pathologic changes in other organs of rats fed the dye.

MATERIALS AND METHODS

One hundred female and one hundred male albino rats of the Sprague-Dawley strain, weighing 120–200 gm. and housed in individual wire cages, were fed *ad libitum* 0.06 per cent *m*′MeDAB in the basal semi-synthetic ration of Griffin, Nye, Noda, and Luck (6). One hundred and forty-six were sacrificed at weekly intervals to study intermediate histologic changes. Fifty-four rats, 27 male and 27 female, were maintained from 15 to 29 weeks until they died of tumor complications, pneumonia, or were killed because they were moribund.

A control group of 48 rats was fed the basal diet without the carcinogen and developed no liver damage.

All animals were examined at autopsy. Liver and bone marrow smears were made for a separate cytological study. In most of the animals all organs were routinely examined histologically, including the hypophysis, brain, thyroid, adrenals, gonads, lumph nodes, and bone marrow, as well as the visceral organs. Tissues were fixed in either Vandegrift's solution (14) or 10 per cent formalin, and were stained routinely with hematoxylin-eosin; special stains were made as indicated. The liver and lungs received particular attention, and all lobes of these were microscopically examined.

Weekly liver punctures, to follow the liver changes and tumor development, were done on seventeen animals without a death. A standard 14-gauge needle was inserted just below the right costal margin into the liver. Aspiration with a 30-cc. syringe delivered liver tissue and blood into the barrel. Crush preparations of tissue bits fixed in Vandegrift's solution (14) (for 5–10 minutes and water washed) were stored in 70 per cent isopropyl alcohol until stained with hematoxylin-eosin.

OBSERVATIONS

Gross pathology.—Gross liver changes first became apparent by the ninth week. The first changes were slight enlargement, a brown-yellow, tense, and finely mottled capsule, with increased friability of the parenchyma. Cysts and nodules of proliferated ducts appeared in the parenchyma. From the twelfth week on, the liver changes advanced progressively, and the gross appearance was that of cirrhosis. By the fifteenth week the earliest liver malignancy, which subsequently caused the death of an animal, had developed. No gross or microscopic changes appeared in the other organs until the twelfth week, when the spleen and periportal and mediastinal lymph nodes were enlarged. No changes except those associated with cachexia were found in other organs at this time or later.

Among the 54 rats surviving from 15 to 29 weeks, 53 developed malignant liver neoplasms, and one developed a benign adenoma. There was no significant difference in tumor types or time of onset between the sexes. Nodular cirrhosis in which one lobe was often more involved than the

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others developed in all except the animal with the adenoma. Thin-walled cysts containing clear, yellow fluid were found both subcapsular and within the parenchyma; in some instances these cysts were so abundant as to convert the cut surface to a honeycomb appearance.

The tumors varied in location, size, number, consistency, and structure, both in different livers and in the same liver. In many instances all lobes contained tumors, although the majority was found in the median lobe, and in no instance was this lobe tumor-free. The two lateral lobes were next most frequently involved, with the caudate lobe least involved. The capsule over the superficial tumors was usually considerably thickened and frequently adherent to the adjacent diaphragm, stomach, pancreas, spleen, or omentum. A testicle was occasionally adherent to the liver. Sometimes a pedunculated liver "polyp" surrounded by capsular connective tissue and attached by a thin connective tissue pedicle was found.

Large tumors measuring as much as 3–4 cm. in diameter, together with many smaller ones, gave the external surface of the liver a rough, nodular appearance. The large tumors contained necrotic centers, often with extensive hemorrhage which occasionally extended through the capsule and was the immediate cause of death. In the smaller tumors there was a variable degree of necrosis but no hemorrhage. The consistency of the tumors varied; some were friable and contained blood cysts; others were firm and cellular; a few were fibrous.

Gross changes found in other organs were enlargement of the spleen, atrophy of the thymus, and a red, moist bone marrow. No particular change was noted in the suprarenals, hypophysis, thyroid, gonads, kidneys, intestinal tract, heart, striated muscles, or brain.

Microscopic pathology.—Between the sixth and ninth weeks cirrhosis became microscopically evident as connective tissue overproduction produced distortion in the architectural pattern of the parenchyma. In the early stages the thin connective tissue septa contained excess fibroblasts, proliferating bile ducts and blood vessels, with an increase of the stromal collagen. As the cirrhosis progressed, the stroma became more irregular, and in the larger septa blood vessels, interstitial macrophages containing canary-yellow inter-cytoplasmic pigment and bile ducts containing a granular brown material became frequent. Multiple cysts of variable size, lined with a single layer of flat or cuboidal epithelium and containing a clear, yellow fluid with yellow, granular amorphous debris were often found in these thickened septal bands.

The tumors originating from the proliferated bile ducts were classified either as benign biliary adenomas or adenocarcinomas of simple or papillary cystic type. The biliary adenomas were lightly invested by connective tissue and were composed of proliferated ducts lined with uniform cuboidal or columnar epithelium. There was no alteration in nuclear-cytoplasmic ratio, and mitotic figures were rare. In contrast, the adenocarcinomas of biliary origin were characterized by a definite alteration of both cellular size and shape. Mitoses were common, and the nuclear-cytoplasmic ratio was increased. Architecturally, these tumors formed both simple and papillary acini lined with irregular columnar cells in two, three, or more layers. Excessive secretory function resulted in complete filling of the acinar lumina with mucus (Fig. 1). Considerable collagenous tissue surrounded the adenocarcinomas, in distinction to its absence or inconspicuous amount about the benign adenomas. This same pattern was also repeated in the distant metastases.

The weekly liver punch biopsies and crush preparations before and at necropsy enabled us to study the cytological changes in the liver cells as the experiments progressed. The first histologic change, observed 4 weeks after beginning the *m'*MeDAB feeding, consisted of enlargement of the liver-cell nuclei with an increase in nuclear chromatin. Regression in cell size, except in isolated clusters, became apparent between the sixth and ninth weeks. The nuclei of the cells in these focal clusters continued to increase in size and to amass chromatin. The nucleoli dispersed, became multiple, and by the twelfth to fourteenth weeks the nuclei had increased to as much as 24 μ in diameter. Liver punctures at this time were followed by growth of liver cancer along the needle puncture tract. Adenomatous hyperplasia, fatty infiltration, and various stages of necrosis in individual cells were found co-existent with cirrhosis. Paraffin tissue preparations confirmed these cytologic observations (Fig. 2).

Our interest became focused on determining the mode of development and the distribution and location of the focal clusters of hyperchromatic giant liver cells which first appeared during the ninth week and were numerous after the twelfth week (Figs. 3 and 4). These cell groups were not confined to any specific portion of the liver or lobule, but occurred in any region. They were believed to be the source of the cancer cells found in the crush preparations, and their transition into tumors was demonstrated histologically.

Five types of malignant neoplasms appeared in the livers in these experiments. Three were parenchymal tumors histologically classified as hepatoma, adenocarcinoma, and anaplastic carcinoma; and two were stromal tumors, classified as fibrosarcoma and angiosarcoma, respectively.

The malignant hepatomas were both large-cell and small-cell types, and numerous architectural variations including solid, acinar, glandular, and papillary-cystic variations were encountered. The cells of the large-cell-type hepatomas were well differentiated, contained abundant acidophilic cytoplasm, and the cell margins were distinct (Fig. 5). Variations in nuclear size and configuration were frequent, and chromatin clumping was conspicuous. Nuclear membranes were distinct, and the nucleus vesicular. Mitoses were uncommon. The cells were arranged in cords juxtaposed to endothelial-lined blood sinusoids and in architecture closely resembled that of normal liver.

The small-cell-type hepatomas were composed of cords of cells in an architectural pattern also resembling that of normal liver. Both acinar groups and syncytial cords were present in the same tumor and in their metastases. Tumor cells appeared to be directly lining the blood sinuses; sinusoidal endothelium, if present, was inconspicuous (Fig. 6). Cell boundaries were indistinct, and the basophilic cytoplasm scanty. Heavy chromatin and absence of vesiculation characterized the nuclei of the small-cell hepatomas, as contrasted to the granular and vesicular nucleus of the large-cell hepatoma. In the small-cell hepatomas mitotic figures were numerous.

Primary adenocarcinomas occurred both adjacent to, apart from, and intermingled with malignant hepatomas. Two histologic variants of these occurred—one a large-cell and the other a small-cell type. Both were composed of cuboidal or columnar epithelial cells, forming either an acinar or a glandular structure. The cells were often multi-layered, and the nuclei were hyperchromatic and variable, both in size and shape. Numerous atypical mitotic figures were seen in most sections (Fig. 7). Papillary and cystic formations were encountered. One of the distinguishing histologic characteristics of these tumors was the abundance of stroma which contained no characteristic blood vascular pattern nor uniformity of pattern.

Anaplastic carcinoma was characterized by small cells containing nuclei filled with dense homogeneous chromatin. The nuclei appeared to occupy most of the cell (Fig. 8). Nucleoli were not readily found, while mitotic figures were numerous. Architecturally, this cancer generally appeared as a group of compact cells surrounding and radiating from a central blood vessel. Neither sinusoidal spaces nor interstitial stroma were visible.

The two types of stromal tumors were classified as fibrosarcoma and angiosarcoma. The fibrosarcomas were composed of compact groups of elongated spindle-cells (Fig. 9). Their nuclei were large, coarsely granular, and mitoses were numerous. The angiosarcomas were composed of disoriented anaplastic cells forming irregular channels. Mitoses were common. In those tumors containing the blood cysts, the neoplastic cells appeared to have unusual invasive potentialities. Both of these sarcomas were occasionally found as metastases.

In four instances well differentiated cartilage, bone, and calcification were also present. In each of these, the animals had been on the experiment longer than 21 weeks.

Metastases.—All types of the liver tumors were found to metastasize to the lungs, lymph nodes, peritoneum, and omentum, while certain types were found only in the diaphragm, spleen, pancreas, retroperitoneal structures, suprarenals, and heart. All modes of metastasis—implantation, direct extension, and spread by venous, arterial, and lymphatic routes—appeared to be involved.

The lungs were the most common site, with metastasis in 40 of 54 rats (74 per cent). All types of the tumors were found metastatic in the lungs. In size, metastases to the lungs varied from pinpoint dimensions to replacement of an entire lobe. These metastases were principally hematogenous. Chronic pneumonia frequently co-existed in these tumorous lungs.

Cervical, mediastinal, and abdominal lymph nodes were routinely examined, but it was difficult to determine grossly metastases in these. Lymph node metastases were confirmed histologically in 25 cases (46 per cent), with the greatest incidence in the periportal lymph nodes. The mediastinal nodes were also involved in some, but in no instances were metastases found in the cervical nodes. The metastases in the lymph nodes generally consisted of all the small-cell types of both the hepatomas and adenocarcinomas, but did not contain the variety of types as found in the lung metastases.

Peritoneal implantation (or metastasis) was found in 47 cases (87 per cent). With few exceptions, gross metastases were found in the omentum, mesentery, and parietal peritoneum. The greatest variation of tumor types occurred in the metastases to the omentum. Microscopically, all variants were found here, as were portions of detached cirrhotic liver. The larger masses often gave

the impression of originating in pedunculated portions of liver parenchyma that had become detached, with autotransplantation to and "imbedding" in the omentum. Spread by direct extension into the thoracic cavity and retroperitoneal structures was obvious in some instances.

Invasion of the diaphragm occurred in 33 cases (61 per cent). In some there was subtotal destruction of the diaphragm with but few muscle bundles remaining. Extension into the hilus of the spleen was generally confined to the capsule, but in three cases the parenchyma, too, was invaded. The pancreas was often the site of tumor invasion by direct extension and by lymphatic infiltration. Likewise, lymphatic extension was often found in the serosa of the duodenum and the stomach. In several instances this invasion extended to the mucosa of these organs.

Other sites included: in one case, metastasis of adenocarcinoma into a suprarenal (Fig. 10); in three others, the wall of the right ventricle and the pericardium were involved. The spread to these unusual sites was apparently of retrograde lymphatic mode.

Other organ changes.—The spleen, lymph nodes, bone marrow, and cirrhotic areas of the liver showed consistent microscopic appearance after 105 days of the experiment. The spleens were enlarged by engorgement of the sinusoids with erythrocytes and phagocytes containing intercytoplasmic pigment, and by reticulum hyperplasia and myeloid metaplasia. The lymph nodes in all regions showed the characteristic picture of sinusoidal distention and engorgement with phagocytes, reticulum hyperplasia, and of follicular regression. The bone marrow was consistently hyperplastic with a predominance of erythroblastic elements. Myeloid metaplasia was present in the bone marrow, spleen, and lymph nodes and was also demonstrable in the cirrhotic and the tumor-bearing areas of the liver. Testicular atrophy in various stages was found in 50 per cent of the male rats of the experiment. The kidneys showed no consistent or noteworthy abnormalities other than a light yellow pigment within the tubular epithelium. The suprarenals, ovaries, hypophysis, thyroid, thymus, salivary glands, and brain showed no significant changes.

DISCUSSION

The 54 rats reported in this study were fed 0.06 per cent 3′-methyl-4-dimethylaminoazobenzene (*m*′MeDAB) *ad libitum* in a basal diet (6) until death or near death, in an effort to determine (*a*) the life span of the tumor-bearing rats, (*b*) the mode of development of the tumors and the time required for carcinogenesis, (*c*) the types of tumor which might be produced, (*d*) the frequency and distribution of metastases, and (*e*) the effects of carcinogen and basal diet feeding upon organs other than the liver in rats.

Mortality was highest between the 19th and 26th weeks. Eighty-four per cent of the entire group died during this period. Only three animals survived 27–29 weeks (Chart 1).

The tumors appeared to arise by transformation of normal liver cells, bile duct epithelium, and the interstitial stroma from 15 to 29 weeks after beginning the feeding of *m*′MeDAB.

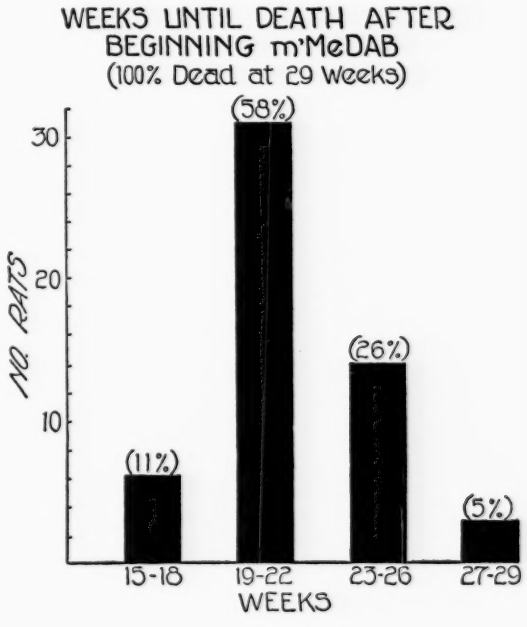

CHART 1

The types of tumors found in the livers were as follows: malignant hepatomas, small- and large-cell types, 51 (94 per cent); anaplastic carcinoma, 7 (12 per cent); adenocarcinoma, liver-cell type, 30 (55 per cent); adenocarcinoma, bile duct type, 38 (70 per cent); sarcoma, 5 (9 per cent). Benign metaplastic changes represented by bone and cartilage formation occurred in 4 (7 per cent). The types of liver tumors produced are shown in Chart 2. Over 90 per cent of the rats had more than one variety of tumor, and in several instances as many as seven different neoplastic growths were present in a single liver.

The incidence of organ metastases is shown in Chart 3. The lungs were involved in 40 (75 per cent); the lymph nodes in 25 (46 per cent); and the peritoneal cavity was invaded in 47 (87 per cent). The relationship of the duration of the dye feeding to the distribution of metastases and the

sites and incidence of metastases is summarized in Table 1.

Feeding of *m'*MeDAB under these conditions resulted in no change in other organs until about the fifteenth week, at which time the bone marrow became hyperplastic and the spleen enlarged by myeloid metaplasia, while in some cases the lymph nodes and liver also contained myeloid elements.

Of interest is the liver body-weight ratio, which

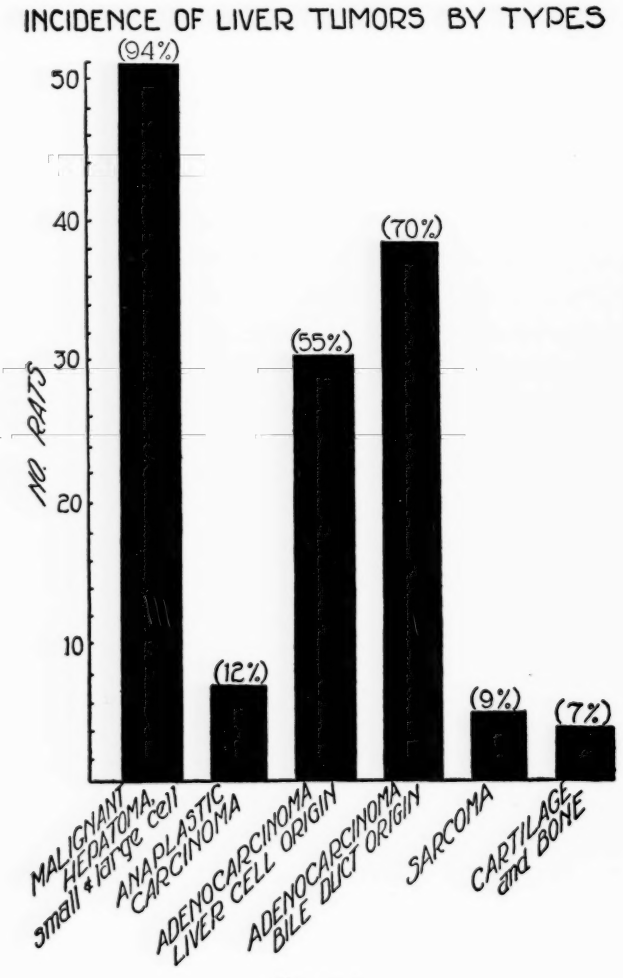

CHART 2

was recorded at the time of autopsy in 19 of the 54 rats. The liver of a 200-gm. rat normally weighs about 10 gm., i.e., the liver body-weight ratio of a normal rat is about 1:20. The average in these experiments of liver body-weight ratio was found to be 1:5, with extremes of 1:2 and 1:9—i.e., some of these livers were very large and extensively replaced with tumor.

Histologically, the results obtained in our experiments were similar to those reported by Kinosita (7, 8), Maruya (9), Orr (12), Edwards and White (4), and Opie (10, 11) in rats fed *p*-dimethylaminoazobenzene. Cortell (3), who used *m'*methyl-*p*-dimethylaminoazobenzene, reported focal neoplasms in the liver and a high incidence of lung metastasis similar to our observations.

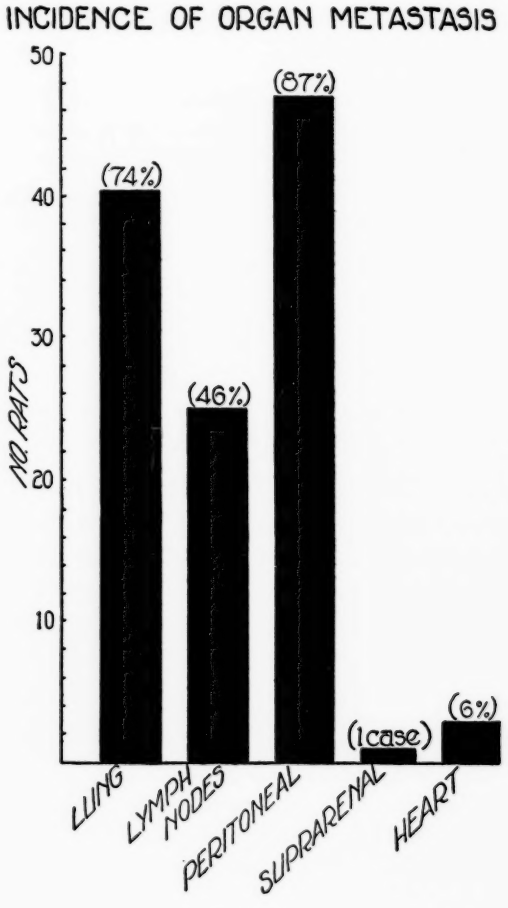

CHART 3

TABLE 1

DISTRIBUTION OF METASTASES IN RATS FED *m'*MeDAB

DURATION OF FEEDING IN WEEKS		METASTASES TO					
		Lung		Lymph nodes		Peritoneal extension	
	No. RATS	No. rats	Per cent with metast.	No. rats	Per cent with metast.	No. rats	Per cent with metast.
15–19	12	6	50	3	25	9	75
20–23	26	23	88	12	46	25	96
24–29	16	11	69	10	63	13	81
TOTAL	54	40	74	25	46	47	87

CONCLUSIONS

1. Two hundred rats of the Sprague-Dawley strain were fed *ad libitum* a basal semi-synthetic diet containing 0.06 per cent *m'*MeDAB. Fifty-four animals were allowed to live 15–29 weeks. Fifty-three of these developed various malignant tumors in cirrhotic livers. One developed a benign adenoma.

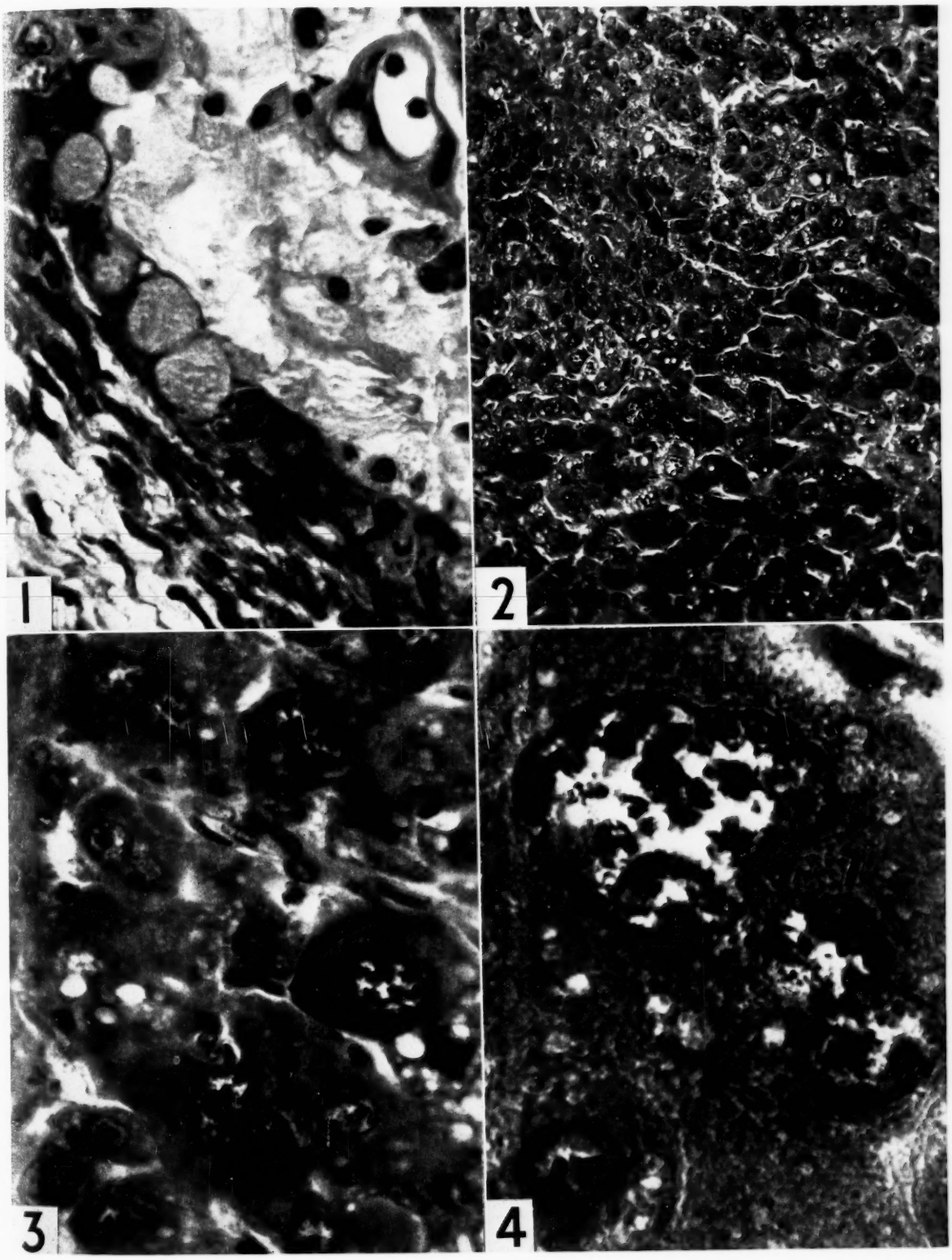
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FIG. 1.—Bile duct adenocarcinoma with mucus production in cells, and filling lumen, after 25 weeks *m'*MeDAB. ×540.

FIG. 2.—Focal variations in size and shape, and chromatin massing in liver cells, after 12 weeks *m'*MeDAB. ×120.

FIG. 3.—Normal liver cells and hyperchromatic cellular clusters, after 12 weeks *m'*MeDAB. ×540.

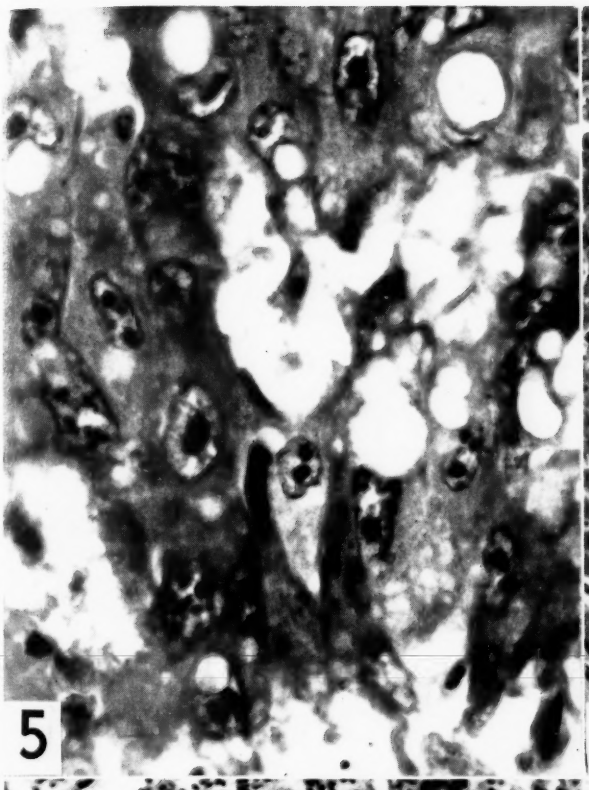
FIG. 4.—Liver cells in clusters undergoing malignant transformation, after 12 weeks *m'*MeDAB. ×1,250.

FIG. 5.—Sinusoidal dilatation within a malignant hepatoma, after 19 weeks *m'*MeDAB. ×540.

FIG. 6.—A nodule of small-cell liver carcinoma in cirrhotic liver. Note normal liver cell groups at the periphery of the malignant nodule, after 20 weeks *m'*MeDAB. ×120.

FIG. 7.—Adenocarcinoma of liver cell type, with blood cysts, after 21 weeks *m'*MeDAB. ×120.

FIG. 8.—Anaplastic liver cell carcinoma, after 26 weeks *m'*MeDAB. ×120.



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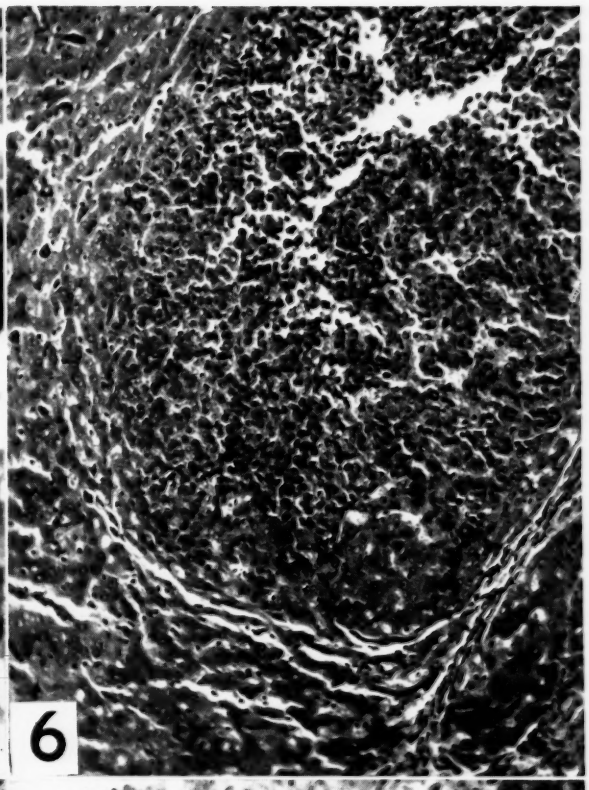

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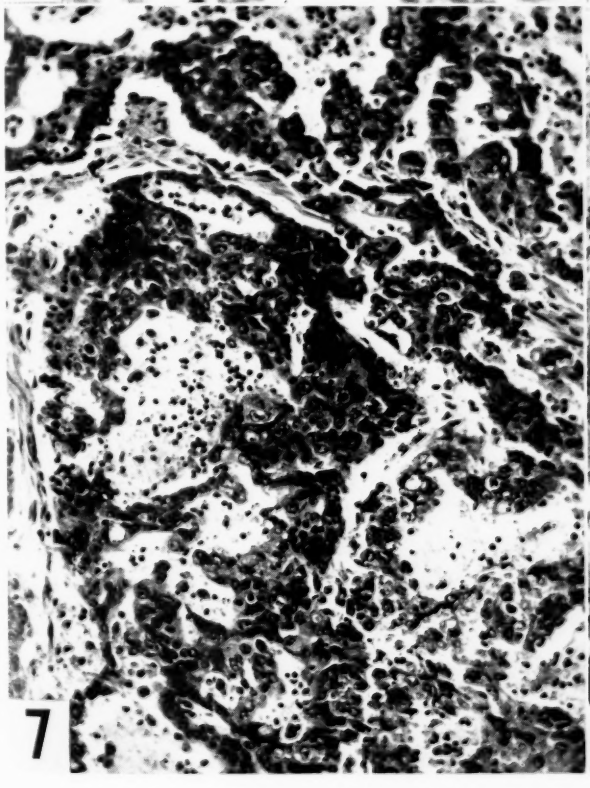

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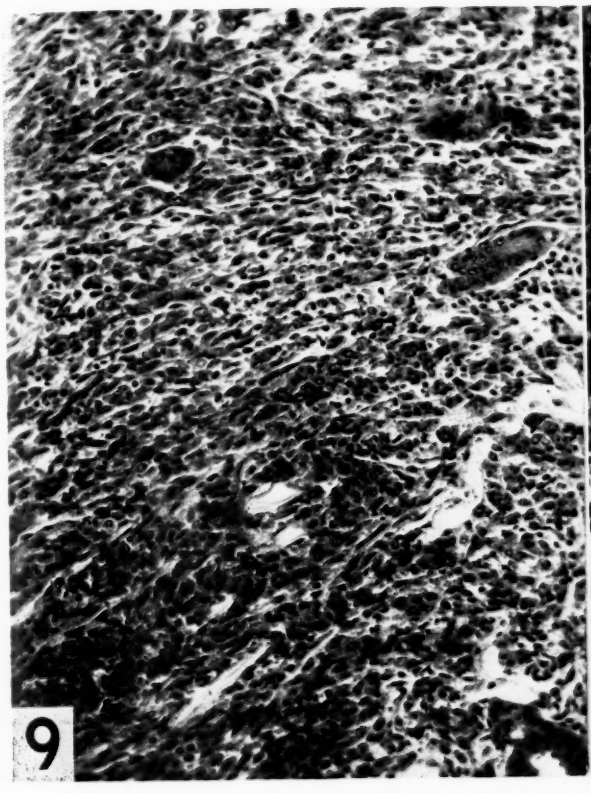
FIG. 9.—Sarcomatous stromal change and residual bile ducts, after 20 weeks *m'*MeDAB. ×120.

FIG. 10.—Liver adenocarcinoma metastatic into adrenal, after 21 weeks *m'*MeDAB. ×120.



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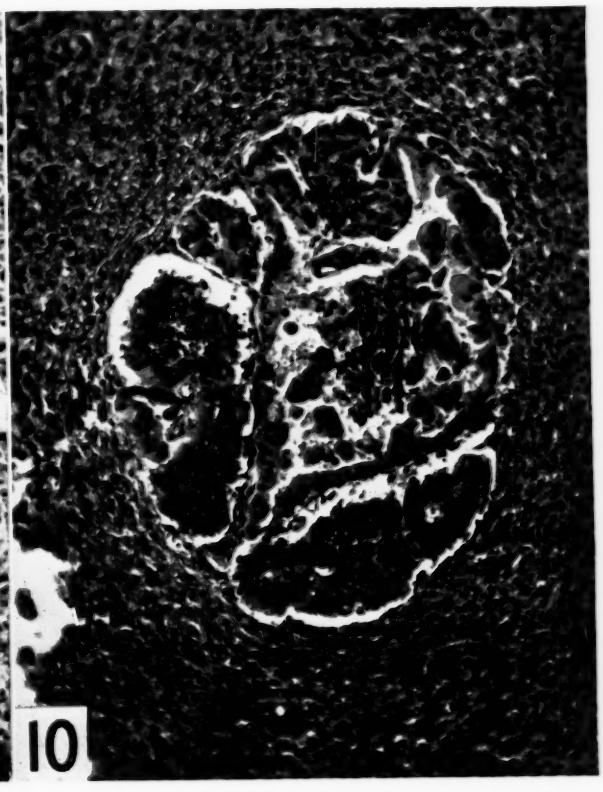

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2. The malignant neoplasms of liver cell origin were (*a*) hepatoma, (*b*) adenocarcinoma, and (*c*) anaplastic carcinoma. The malignant bile duct carcinomas were (*a*) adenocarcinoma and (*b*) papillary cystadenocarcinoma. Malignant neoplasms of stromal origin were (*a*) fibrosarcoma and (*b*) angiosarcoma.

3. Metastasis was observed in the lungs in 74 per cent, the peritoneal cavity in 87 per cent, to the lymph nodes in 46 per cent, to the heart in 4 per cent, and the adrenal gland in 2 per cent.

4. Other changes observed were hyperplasia of the bone marrow in all, and myeloid metaplasia in the liver, spleen, and lymph nodes.

ACKNOWLEDGMENTS

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Alteration of the Mitochondrial Pattern in the Liver of Tumor-bearing Mice

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The increased percentage of water in the liver of tumor-bearing animals, as reported by McEwen and Haven (6), suggests that it may be related to some structural changes in the cytoplasmic pattern of the cell. The investigations of Opie (9) demonstrated that osmotic changes inside the cell are related to alteration of mitochondria. Attempts have thus been made to study mitochondria in the liver of tumor-bearing mice.

METHODS

Histological studies were performed on mitochondria in the livers of mice bearing malignant tumors. A total of 30 mice was used in the experiment. The tumors were: transplanted fibrosarcoma of the albino mouse, 9 animals; Meyer tumor of the albino mouse (a mammary tumor of Brazilian origin), 5 animals; spontaneous and transplanted mammary carcinoma of the C3H strain mouse, 3 animals. The livers of 5 albino and 5 C3H strain mice served as control. All animals had been kept on an *ad libitum* stock diet (1). The mice were killed while food was available in order to avoid alteration of the mitochondria by the lack of food. Animals bearing open necrotic tumors were discarded. Tumors of sacrificed animals were prepared and weighed separately. In order to correlate the size of the liver with changes in mitochondria, the ratio liver/body was determined in each case, i.e., the gram value of the liver related to 1 gm. of body, deprived of its tumor.

Pieces of liver were fixed in Regaud's fluid. Histological sections of 3 μ were stained according to Altman's method. Ultraviolet light proved the best illumination for studying mitochondria microscopically, since acid-fuchsin-stained mitochondria absorb ultraviolet light and are thus clearly defined. Sections of liver from tumor-bearing animals were compared with sections of liver from healthy animals.

RESULTS

Noël (8) and Dalton and Edwards (3) describe mitochondria in the normal mouse liver as "varying from long tenuous filaments near the central vein to spheres and plump rods at the periphery of the lobule." Our observations agree with theirs, although the distribution of these forms is not a sharp one, since in some instances spherical mitochondria are also encountered in the vicinity of the central vein and filamentous mitochondria at the periphery of the lobule (Figs. 1, 2).

In the liver of mice bearing tumors of a medium size and with a slightly elevated liver/body ratio, spherical mitochondria are more abundant and extend most frequently into the region of the central vein. In general, they appear enlarged and deeply stained (Figs. 3, 4).

If the tumor is very large and the liver/body ratio is particularly high (0.080), almost no filamentous or rodlike mitochondria are present, and only fine granular and enlarged spherical mitochondria, the latter showing a great variety in shape, are observed (Figs. 5, 6).

DISCUSSION

In the living organism where chemical processes are closely related to the structural elements of the cell, systemic alterations are not merely superficial but extend into the region of cellular morphology. Thus, for instance, the increased water content in the liver of tumor-bearing rats may be related to chemical processes, but at the same time, by its osmotic effect, it also alters the cytoplasmic pattern of the cell.

According to Opie (9), mitochondria are characterized by their behavior as discrete particles with surface properties that undergo changes in the presence of water. They are also very sensitive to any influence or injuries affecting the cell. However, since an important part of the metabolic enzyme system resides in mitochondria, it is possible that any change in their physical structure is accompanied by alterations in the enzymatic reactions related to them.

Studies on normal mouse liver (Noël [8] and Dalton and Edwards [3]) indicate that spherical mitochondria predominate in the peripheral part of the lobule, while in the less active zone, near

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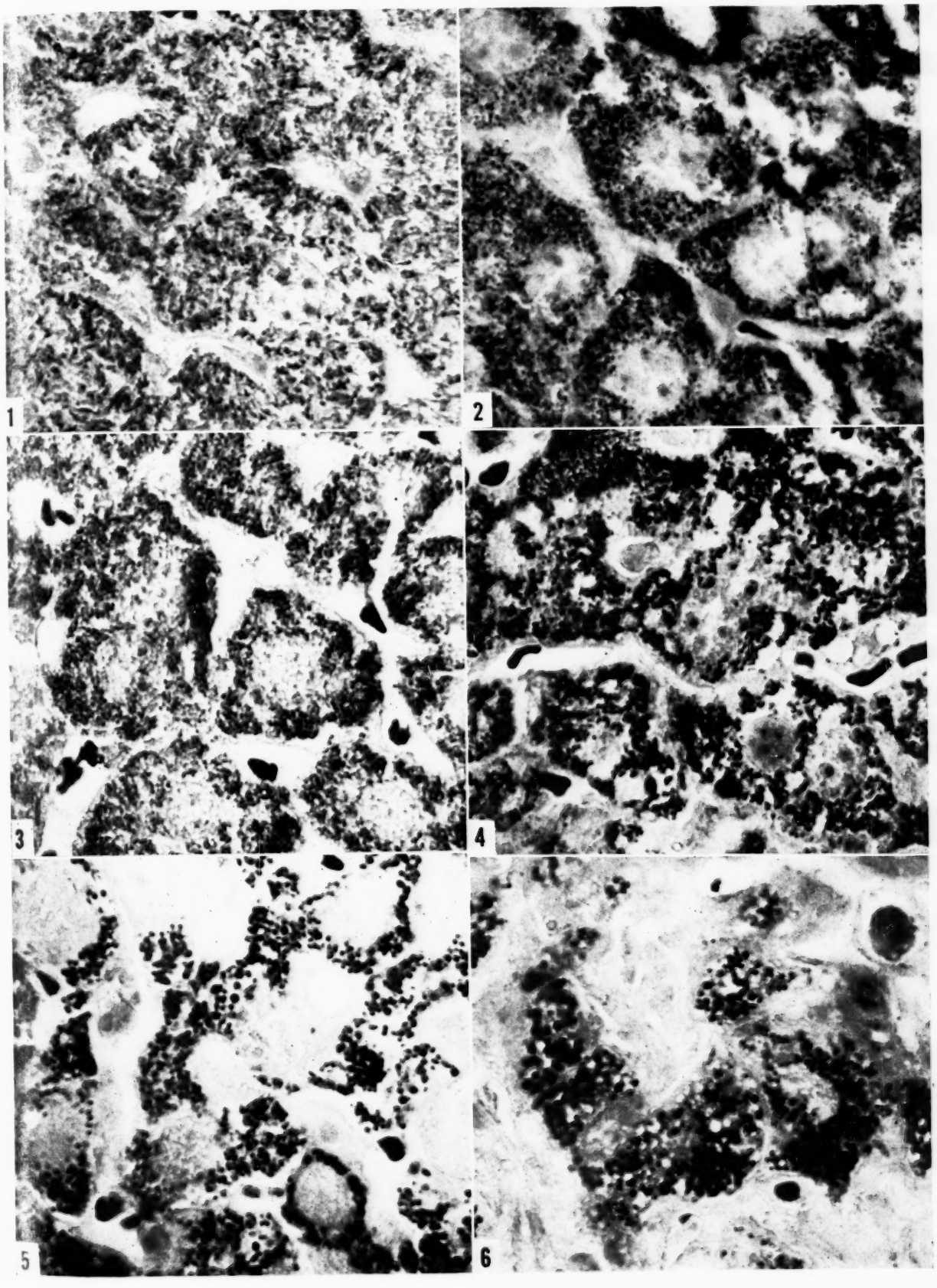
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FIG. 1.—Liver of normal albino mouse, showing predominantly filamentous mitochondria near the central vein. Ratio liver/body 0.049. (Altman's method, ×1,200.)

FIG. 2.—Liver of normal C3H strain mouse showing spherical mitochondria near the central vein. Ratio liver/body, 0.052. (Altman's method, ×1,200.)

FIG. 3.—Liver of albino mouse bearing transplanted fibrosarcoma. It shows predominantly spherical mitochondria near the central vein. Weight of tumor 2.15 gm; ratio liver/body, 0.055. (Altman's method, ×1,200.)

FIG. 4.—Liver of C3H strain mouse bearing transplanted mammary tumor. It shows plump spherical mitochondria near the central vein. Weight of tumor, 1.2 gm.; ratio liver/body, 0.052. (Altman's method, ×1,200.)

FIG. 5.—Liver of albino mouse bearing large transplanted fibrosarcoma. Enlarged and well separated mitochondria are evident. Their number is also diminished. Weight of tumor 16.5 gm.; ratio liver/body, 0.080. (Altman's method, ×1,200.)

FIG. 6.—Liver of albino mouse bearing large transplanted Meyer mammary tumor, showing enlarged spherical mitochondria of different size and shape. Many of the cells are degenerating and do not contain mitochondria. Weight of tumor, 13 gm.; ratio liver/body, 0.080. (Altman's method, ×1,200.)

the central vein, filamentous mitochondria are in the majority. These observations, mainly confirmed by us, served as a general pattern of comparison in the consequent studies (Figs. 1, 2).

The mitochondrial pattern in the liver of tumor-bearing mice presents a different aspect from that of normal mice (Figs. 3–6). If the tumor is not too large and the weight of the liver of the host animal is only moderately increased, which is represented by a slight elevation of the liver/body ratio, the number of filamentous mitochondria is diminished, and spherical forms are abundant even in the vicinity of the central vein. In some of the cells plump, irregular spheres are also recognizable. In general, mitochondria seem to absorb more stain. If the suggestion of Noël is correct, one may conclude that in this stage of tumor development the mitochondrial pattern represents a higher degree of activity than in the control animal.

On the other hand, if the weight of the tumor increases to at least half of the body weight, resulting in a remarkably heavy liver, i.e., high liver/body ratio, almost no filamentous or rodlike mitochondria are visible. Mitochondria appear as fine granules or plump spheres, obviously separated from one another. Their number is also significantly diminished in many of the cells. Many of them clump, forming oversized irregular bodies which stain differently (Figs. 5, 6).

Since a reduced food intake is noted among animals bearing large tumors, one may suppose that the diminution in mitochondria is a consequence of starvation, as demonstrated on rats by Lagerstedt (5) and recently by Muntwyler *et al.* (7). That the decrease in the number of mitochondria is not the consequence of a reduced food intake of the host animal, or at least not a predominant one, seems to be supported by the fact that all host animals develop liver hypertrophy proportional to the size of tumors. A reduction of food intake, however, produces a well defined decrease in the size and weight of the liver that more or less parallels the general loss of the body weight.

The entire mitochondrial pattern of the liver cells reveals a high degree of injury which, according to Cowdry (2), is caused by the administration of some chemical agents or by different kinds of diseases.

If, however, the concept of Yeakel and Tobias (10) is correct, that the liver of animals bearing large tumors present a picture of "work-hypertrophy," the obviously altered mitochondrial pattern in the liver may be explained as the result of extreme hyperactivity. Possibly all these influences are acting on the liver of the tumor-bearing host and elicit the systemic effect suggested by Greenstein (4).

SUMMARY

The mitochondrial pattern in the liver of tumor-bearing mice has been compared to that of healthy animals. A definite change occurred in the shape and size of mitochondria of the liver cells of mice bearing either spontaneous or transplanted carcinomas or transplanted sarcomas, as compared to those of control animals. The greatest alteration was found in the liver of mice bearing the largest tumors and presenting an extremely high liver/body ratio.

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Steroid Hormones and Tumor-Host Relations*

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It has been demonstrated that testosterone propionate in oil causes an increase in hemoglobin and liver catalase activity in the normal rat but that the administration of the steroid in large doses could not overcome the characteristic anemia and loss of liver catalase activity in the tumor-bearing rat (2). It was of interest to see if these effects might be moderated by the administration of a more potent androgen.

The use of pellets of testosterone propionate provided another approach to the problem. Implanted in the subcutaneous tissues, these would give a slow steady absorption of the hormone which might be of greater effect than daily injections in oil.

A pattern of the systemic effects of tumors has been described and evidence presented to suggest that rats in the terminal stage of cancer may be in a state of adrenal cortical insufficiency (3). If this reasoning were valid, it might be anticipated that an exogenous supply of hormones of the adrenal cortex would modify the response of the host to the tumor.

METHODS

As test animals, a group of young, male Sprague-Dawley (Holtzman) rats bearing intramuscular grafts of the Walker 256 carcinoma in both thighs was used. On the ninth day of growth, when the tumors had attained a diameter of 10 mm., injections were begun or pellets implanted. The animals were maintained on Purina Fox Chow and tap water in a room controlled to 72°–78° F.

Testosterone cyclopentylpropionate[1] (TCP) was given in two 5.0-mg. doses, one on the ninth and one on the fourteenth day of tumor growth. Lipo-Adrenal Cortex[2] (LAC) was administered in daily injections of 0.5 ml., increasing to 1.5 ml., with different groups receiving 240, 360, and 420 rat units as total dose. Control animals were given appropriate amounts of cottonseed oil. Pellets of testosterone propionate[3] (TPP) were implanted over the scapular region, five pellets per rat, each placed at a discrete site. At autopsy the pellets were removed, dried, and reweighed for the determination of total absorption.

Hemoglobin was estimated on tail blood the afternoon preceding sacrifice on the twentieth day of tumor growth, control rats being killed at the same time. The two androgen groups were given access to food and water until the end of the experiment, but the LAC group was starved for 16 hours and given 400 mg. glucose intraperitoneally 2 hours before sacrifice.

Tissues were removed under nembutal anesthesia and analyzed by methods previously described (3).

Tumors were measured in two diameters of each tumor and are expressed as the mean of the four diameters. In these experiments the tumor weight corresponds to approximately 25 per cent of the body weight.

RESULTS

Testosterone cyclopentylpropionate.—Table 1 indicates that the only significant difference between the control and treated groups is increased thymus atrophy in the rats given TCP. The mean adrenal weight is smaller in the treated group, but the variation considerable; a similar response is noted in adrenal cholesterol. The failure to influ-

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[1] Testosterone cyclopentylpropionate was provided by the Upjohn Co. through the kindness of Dr. H. F. Hailman. The Upjohn Co. report that this compound has a more potent and prolonged androgenic activity than testosterone propionate.

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[2] Lipo-Adrenal Cortex is an extract of hog adrenals dissolved in vegetable oil, prepared by the Upjohn Co. It is assayed at 40 rat units per milliliter by the survival-growth method, and is equivalent to 2 mg. of Compound E by the muscle work test.

[3] Pellets of testosterone propionate were provided by the courtesy of Dr. B. L. Frank of the Ciba Pharmaceutical Company (Canada). They weighed approximately 15 mg. each and were known to give an absorption of about 0.2 mg. per pellet per day.

ence hemoglobin and liver catalase activity is evident.

Testosterone propionate pellets.—As indicated in Table 2, there is no apparent effect on adrenal ascorbic acid, but adrenal weight and cholesterol are both significantly lower in the treated group. A marked involution of the thymus was noted in the pellet-treated rats, but no significant effect on liver catalase activity or hemoglobin. The average absorption of testosterone propionate from the pellets was 0.99 mg/rat/day.

Lipo-Adrenal Cortex.—Table 3 demonstrates that the injection of adrenal cortical extract produced significant changes only in thymus weight, hemoglobin, and body weight. The mean weight of the two groups was the same at the beginning of the injections. It is difficult to interpret the decreased gain in body weight in the treated group, as the animals were not tube-fed, and the reduction in weight gain may be a reflection of a decreased food consumption. The characteristic loss of sudanophilia in the adrenals of tumor-bearing rats was not as marked in the treated as in the control group. Tumor growth was not inhibited by this treatment, and histological examination of the tumors from the treated animals did not reveal any variation from the control group.

DISCUSSION

The magnitude of a systemic effect is related to the size of the tumor (3). For this reason it is essential in an attempt to modify systemic effects that there be no difference in the growth rate and size of the tumors in the control and treated groups.

TABLE 1

EFFECT OF TESTOSTERONE CYCLOPENTYLPROPIONATE ON TUMOR-BEARING RATS

	Control*	TCP*	P†
Body weight, gm.	245.1 ± 7.3(10)	242.8 ± 8.4(9)	>0.05
Tumor diameter, mm.	39.0 ± 1.0(10)	39.1 ± 1.3(11)	>0.05
Adrenal weight, mg.	28.5 ± 0.7(8)	26.5 ± 1.2(11)	>0.05
Thymus weight, mg.	193.4 ± 20.7(8)	105.2 ± 14.0(11)	<0.01
Hemoglobin, gm/100 ml	8.03 ± 0.44(10)	7.67 ± 0.72(11)	>0.05
Liver catalase, K×10⁴	1,964 ±142(10)	2,053 ±213(11)	>0.05
Adrenal cholesterol, mg/100 mg	2.24 ± 0.28(8)	1.73 ± 0.20(11)	>0.05
Adrenal ascorbic acid, mg/100 mg	0.315± 0.029(8)	0.315± 0.017(11)	>0.05

* Number of observations in parentheses; ± standard error of the mean.
† Probability in *t* test: <0.05 = significant; <0.01 = highly significant.

TABLE 2

EFFECT OF TESTOSTERONE PROPIONATE PELLETS ON TUMOR-BEARING RATS

	Control*	Pellets*	P†
Body weight, gm.	245.1 ± 7.3(10)	230.2 ± 8.9(11)	>0.05
Tumor diameter, mm.	39.0 ± 1.0(10)	38.6 ± 1.0(11)	>0.05
Adrenal weight, mg.	28.5 ± 0.7(8)	25.5 ± 0.9(11)	<0.05
Thymus weight, mg.	193.4 ± 20.7(8)	50.8 ± 4.6(11)	<0.01
Hemoglobin, gm/100 ml	8.03 ± 0.44(10)	8.39 ± 0.70(11)	>0.05
Liver catalase, K×10⁴	1,964 ±142(10)	2,309 ±118(11)	>0.05
Adrenal cholesterol, mg/100 mg	2.24 ± 0.28(8)	1.09 ± 0.23(10)	<0.01
Adrenal ascorbic acid, mg/100 mg	0.315± 0.029(7)	0.306± 0.018(11)	>0.05

* Number of observations in parentheses; ± standard error of the mean.
† Probability in *t* test: <0.05 = significant; <0.01 = highly significant.

TABLE 3

EFFECT OF LIPO-ADRENAL CORTEX ON TUMOR-BEARING RATS

	Control*	LAC*	P†
Body weight, gm.	323.4 ± 7.2(11)	299.3 ± 5.1(12)	<0.05
Tumor diameter, mm.	46.3 ± 2.0(12)	45.8 ± 1.2(12)	>0.05
Adrenal weight, mg.	29.4 ± 1.6(11)	27.4 ± 1.5(10)	>0.05
Thymus weight, mg.	207.6 ±17.6(11)	88.0 ± 6.9(10)	<0.01
Hemoglobin, gm/100 ml	8.5 ± 0.4(11)	10.4 ± 0.5(12)	<0.01
Liver catalase, K×10⁴‡	933 ±94(11)	759 ±123(10)	>0.05
Adrenal cholesterol, mg/100 mg	2.64 ± 0.30(11)	3.28 ± 0.44(10)	>0.05
Adrenal ascorbic acid, mg/100 mg	0.314± 0.016(11)	0.291± 0.015(10)	>0.05
Liver glycogen, mg/100 mg§	0.44 ± 0.05(11)	0.45 ± 0.06(10)	>0.05

* Number of observations in parentheses; ± standard error of the mean.
† Probability in *t* test: <0.05 = significant; <0.01 = highly significant.
‡ Determined on a liver extract prepared by grinding with sand in a mortar. This procedure gives lower results than an extract prepared in a Waring Blendor, as in the androgen experiments, but the ratio of the activity in the livers of control and tumor-bearing rats is the same in both methods.
§ Expressed as glucose, 2 hours after 400 mg. glucose intraperitoneally.

Steroid hormones have been shown to affect the growth of mammary tumors in the human and the response of the host to the tumor (11). In a rat bearing the Walker 256 carcinoma there is no effect on the tumor and only a slight influence on tumor-host relations. Haddow has directed attention to this discrepancy of the effect of chemotherapeutic agents in the laboratory and in the clinic (5).

Although TCP is a more potent androgen than testosterone propionate, this does not necessarily mean that it would have a greater effect on hemoglobin and liver catalase activity. Studies on the metabolic effects of the androgenic hormones have not yet established a correlation between action on enzyme systems and protein synthesis and the effects on the seminal vesicles and prostate of the castrate, immature rat. Kochakian has demonstrated that some steroids with a very weak or absent androgenic activity may have a moderate influence on alkaline phosphatase and thymus involution (8).

The thymus involution in tumor-bearing rats implanted with pellets of testosterone propionate is the greatest that has been observed. The gland is reduced in most instances to a small thread-like structure. This marked involution is produced by the absorption of 1 mg. per day from the pellets and exceeds that resulting from the injection of 2 mg. a day of the same steroid in oil.

The degree of adrenal hypertrophy was reduced by pellets of testosterone propionate, and it might be inferred that the release of ACTH from the pituitary was inhibited by testosterone (1,4). If this were true, an increase in adrenal ascorbic acid might have been expected but was not demonstrated.

The extract of adrenal cortex was able to reduce the degree of anemia but had no influence on the loss of liver catalase activity. It has been reported that an aqueous extract of the adrenal increases hemoglobin in the rat (12).

There appears to be some variation in the response of tumors to steroids of the adrenal cortex (10), but an inhibition of growth of the Walker 256 carcinoma has been reported (7). This occurs in the presence of an inhibition of body growth more marked than in this series.

The failure of LAC to affect the adrenal may not have been evident because of the design of the experiment. The mean weight of the adrenal in the treated group is smaller, and the cholesterol content higher, than in the control. If the series were expanded to include larger numbers of animals, the differences might become significant. Such a possibility is supported by the fact that LAC did have a sparing action on the loss of adrenal sudanophilia, which has been correlated with cholesterol content (9).

The failure to produce anticipated effects may be more fundamental, and concerned with an inadequate dosage of LAC. It has been estimated that the daily output of the adrenals in the rat is the equivalent of 25 ml. of an aqueous extract of the adrenal cortex, based on the amount required to permit normal performance of the work test in the adrenalectomized rat (6).

SUMMARY

Pellets of testosterone propionate reduce adrenal hypertrophy in the tumor-bearing rat, and an extract of the adrenal cortex diminishes the degree of anemia. No effect was noted on the growth of the Walker 256 carcinoma.

ACKNOWLEDGMENTS

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Systemic Effects of Tumors in Force-fed Rats*†

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The concept of a tumor as a nitrogen trap has been developed and evidence presented that, with the host eating *ad libitum*, there is a movement of nitrogen from the carcass to the tumor (10).These experiments would indicate that the primary effect of the tumor on the host is the production of anorexia and that altered nitrogen metabolism is a secondary factor. In view of this it seemed of interest to force-feed tumor-bearing rats, and to study nitrogen and sodium chloride excretion and the presence or absence of characteristic systemic effects.

METHODS

Young male Sprague-Dawley[1] rats of the same age and weight received subcutaneous grafts in both lumbar regions of a suspension of the Walker 256 carcinoma, under aseptic conditions. On the next day feeding of the high fat diet of Ingle (8) was begun by stomach tube, the rats being brought to full feeding on the fifth day, when they received two feeds of 13 ml. each. At this time they were placed in metabolic cages and force-fed twice a day, distilled water being available at all times. The urine was collected in bottles containing citric acid and a small crystal of thymol. The rats were weighed daily and the tumors measured at frequent intervals.

The 24-hour urine samples were analyzed for nonprotein nitrogen (12), sodium (11), and chloride (2). At intervals total nitrogen was determined on an aliquot of the food to ensure homogeneity. The animals were housed in an air-conditioned room with thermostatic temperature control. Twice-daily temperature records gave a mean of 25 ± 1° C.

When the tumors had attained a large size, the rats were killed by cervical dislocation after hemoglobin had been determined on tail blood (6) and they had fasted 16 hours. A fragment of liver was disintegrated in the Waring Blendor and catalase determined by a titrimetric procedure (7). The remainder of the liver was used for the determination of moisture by drying at 110° C., total nitrogen by the Kjeldahl method, and total lipid by extraction with ether in the Soxhlet apparatus. The adrenals were weighed on a torsion balance and fixed in formalin for staining with Sudan IV (4).

Total body weight of the rats was determined just before they were killed, and the tumors removed after death and weighed. Carcass weight was determined by subtracting the tumor weight from the total body weight.

RESULTS

In the study of force-fed rats in metabolic cages, the experiments have been conducted on groups of four rats, two control and two tumor-bearing. The plotted values in the figures to be presented are the means of the two control and two tumor-bearing rats. Since the absolute level of excretion may vary from group to group, the data have not been pooled, but representative figures are given which have been reproduced in several experiments.

TABLE 1

TUMOR GROWTH IN FORCE-FED RATS

Time in days	Mean tumor diameter* (mm.)	Tumor weight† (gm.)
4	13.7	2.9
7	21.2	11.3
10	30.0	31.2
12	36.7	54.3

* Single tumor.
† Total of both tumors.

The size of the tumors from the zero time when the animals were placed in the metabolic cages are given in Table 1, along with the tumor weights calculated by Schrek's formula (13).

The mean body weights of the rats are plotted

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† A preliminary report was presented to the Fifth International Cancer Congress, Paris, July, 1950.

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[1] Obtained from the Holtzman-Rolfsmeyer Co., R. 4, Madison, Wis.

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against time in Chart 1, the carcass weight of the tumor-bearers being corrected according to Schrek's formula. It is evident that in the force-fed tumor-bearer there is not the loss of carcass weight found by Mider *et al.* under the conditions of their experiments. This is supported by a comparison of the final body weights of the controls with the carcass weights of the tumor-bearers in Table 2.

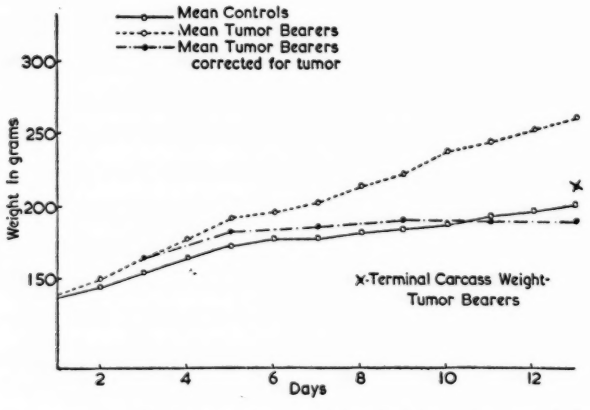

CHART 1.—Body weight of tumor-bearers, corrected for tumor weight.

TABLE 2

BODY WEIGHTS OF FORCE-FED RATS

(gm.)

Type of rat	Initial weight	Carcass weight	Gain
Run no. 3:			
Control 1	135	226	91
Control 2	137	244	107
Tumor B 1 (20 per cent)*	136	247	111
Tumor B 2 (24 per cent)*	137	235	98
Run no. 4:			
Control 1	220	246	26
Control 2	224	250	26
Tumor B 1 (23 per cent)*	219	245	26
Tumor B 2 (22 per cent)*	214	238	24

* Figures in parentheses express tumor weights as per cent of total body weight.

The urinary nonprotein nitrogen excretion is plotted in Chart 2. The nitrogen retention which is evident persisted until the animals were killed. The nitrogen intake of 420 mg. of nitrogen per day, determined by an analysis of a 26-ml. aliquot of the diet, indicates that the rats were in a constant positive nitrogen balance.

Daily excretion of sodium and chloride in normal and tumor-bearing rats are presented in Charts 3 and 4. It is apparent that a retention of sodium chloride does occur in the tumor-bearing animal.

Hemoglobin values, liver catalase activity, and adrenal weights are presented in Table 3, demonstrating that systemic effects are produced in the host, even when carcass weight is maintained. The characteristic loss of sudanophilia has been observed in the adrenals of the tumor-bearing rats.

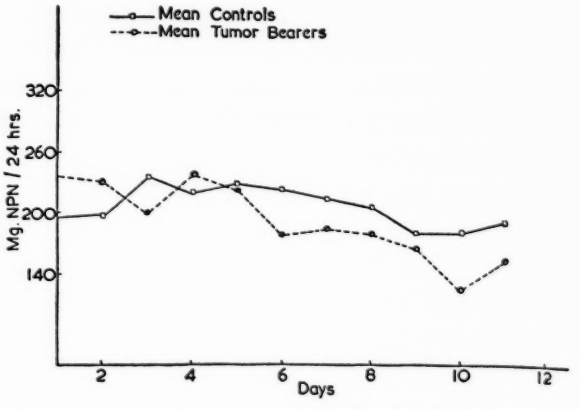

CHART 2.—Urinary nonprotein nitrogen excretion per 24 hours.

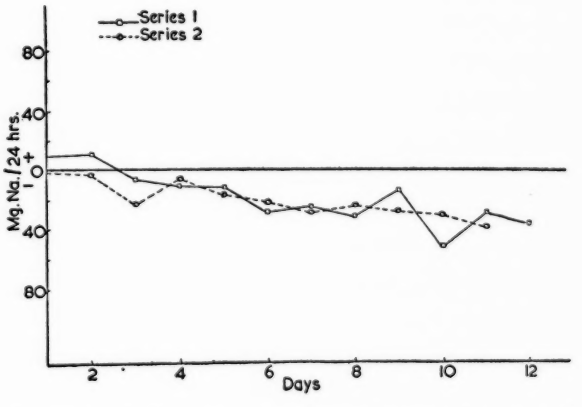

CHART 3.—Sodium excretion, tumor-bearer minus control

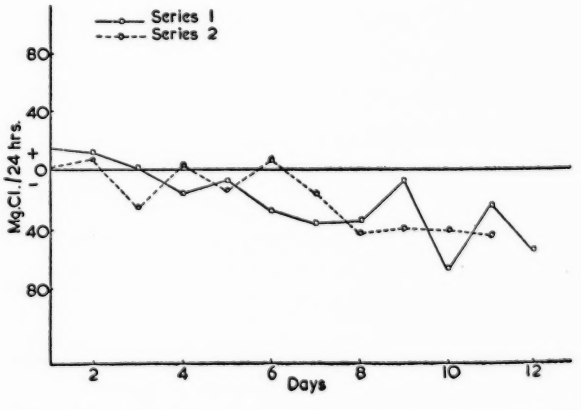

CHART 4.—Chloride excretion, tumor-bearer minus control

The anemia is actually more severe than would appear from Table 3. The tumor-bearing rats on the high fat diet develop a lipemia, which is not present in the control rats fed the same diet. Owing to this lipemia, the transmission of light in the photoelectric colorimeter is diminished, giving

higher values than are due to the amount of hemoglobin present. In conjunction with this lipemia there is an increase in the lipid content of the livers of the tumor-bearing rats, as well as an increase in moisture and nitrogen (Table 4).

TABLE 3

SYSTEMIC EFFECTS IN FORCE-FED TUMOR-BEARING RATS

Type of rat	Hemoglobin (gm/100 ml)	Catalase (K×10⁴)	Adrenal weight (both in mg.)
Control 1	15.8	4,300	42.8
Control 2	15.5	3,900	37.9
Tumor B 1	10.1	2,060	98.5
Tumor B 2	11.3	2,300	80.4

TABLE 4

ANALYSIS OF LIVERS OF FORCE-FED RATS

	Control (2)	Tumor bearer (2)
Per cent moisture	68.0	73.0
Per cent lipid	17.3	37.3
Mg N/gm dry wt	87.0	92.5
Mg N/gm fat-free dry wt	120.0	161.5

It may be noted that in one experiment tumor-bearing rats received implants of pellets of testosterone propionate. The excretion of nitrogen, sodium, and chloride in these rats was at the same level as that observed in untreated tumor-bearers.

DISCUSSION

Under the experimental conditions of forced feeding, whereby anorexia is overcome, the tumor-bearing rats do not lose carcass weight but develop typical systemic effects, such as loss of liver catalase activity, anemia, adrenal hypertrophy, and loss of adrenal sudanophilia (3).

This might be considered as evidence against the concept of the nitrogen trap playing a major role in the lethal effect of tumors (10) and the suggestion that liver catalase effects might be the result of malnutrition of the host (5). Before these theories are discarded it will have to be established that there has not been a nitrogen loss from the carcass in these experiments and that the observed systemic effects are responsible for the lethal effect of a tumor.

It is possible that nitrogen may have been lost from the carcass and replaced by fat. The experimental animals exhibited the increase in liver nitrogen which is noted when carcass nitrogen is being surrendered to the metabolic pool (14).

The data in Table 4 suggest that the dry liver of the tumor-bearing rat is essentially lipid and protein, but these factors do not account for the total dry weight of the control liver, the balance presumably being mostly glycogen. Increased liver lipid has been reported in tumor-bearing mice, but only after a 48-hour fast (1).

It is not surprising that tumor-bearing rats on an adequate dietary intake retain more nitrogen than normal controls, since they are synthesizing additional protoplasm. The retention of sodium and chloride may be explained partly by the increased moisture content of the liver (9) and the high moisture content of the Walker 256 carcinoma (14), but this does not account for all the salt retained.

It will be of interest to determine the effects of high carbohydrate and high protein diets on the tumor-bearing rat and to delineate the minimum dietary intake required to prevent weight loss from the carcass of a tumor-bearing rat.

SUMMARY

Tumor-bearing rats force-fed a high fat diet retained more nitrogen, sodium, and chloride than did control rats.

The force-fed rats bearing tumors did not lose carcass weight, but developed anemia and exhibited the typical enlarged adrenals and loss of liver catalase activity.

It is suggested that the loss of carcass weight in tumor-bearing animals is not a necessary component of the reaction leading to the development of systemic effects.

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The Pathogenesis of Uterine Lesions in Virgin Mice and in Gonadectomized Mice Bearing Adrenal Cortical and Pituitary Tumors*

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Earlier papers from this laboratory have reported that, in certain strains of mice which develop adrenal cortical hyperplasia or cortical neoplasms following early gonadectomy, the accessory reproductive organs show evidence of hormonal stimulation. In the DBA strain, adrenal cortical hyperplasia appears between 2 and 6 months of age and is followed by growth of the uterus, vagina, and mammary glands at 7–12 months (6). In females of the CE strain, carcinomas develop in the areas of adrenal cortical hyperplasia in 100 per cent of the animals by 6–12 months of age, and growth of the accessory reproductive organs begins about the eighth month (24, 25). Transplantation of adrenal tumors into gonadectomized animals is followed by uterine growth. This would indicate that the abnormal adrenal cortex is the source of the steroid hormones which stimulate the reproductive tract (23, 28). A more recent study of various F_1 hybrids has shown the occurrence of nodular hyperplasia which progresses to carcinoma of the adrenal cortex following early gonadectomy (4). These adrenal changes are followed first by growth of the accessory reproductive organs and later by the appearance of basophile nodules of the anterior pituitary.

It has been observed that the uteri of gonadectomized females of the DBA and CE strains frequently show histologic abnormalities (26)[1] which are also found in gonadectomized DBA × CE reciprocal hybrid females.[1] Similar observations were made by Smith (17), who noted cystic and other changes in the uteri of C3H and C3H × A hybrid gonadectomized females with adrenal cortical hyperplasia, adenomas, and adenocarcinomas. We have found uterine abnormalities not only in gonadectomized females with structural changes in the adrenal cortex but also in intact virgin females.

The present study is designed to elucidate the pathogenesis of the uterine lesions in both intact virgin and gonadectomized females of the F_1 cross between DBA female and CE male and the reciprocal cross. The findings and the pathological and endocrinological conclusions drawn from them are to serve as a basis for histochemical studies on similar mice.

MATERIALS AND METHODS

A total of 101 mice was used in this study. Fifty-one animals were of the DBA female × CE male F_1 cross. The remaining 50 mice were the offspring of the reciprocal F_1 cross between the parent strains. Approximately one-half of each group of hybrids was gonadectomized from 1 to 3 days after birth. The intact females were isolated from males at weaning and were kept as virgin controls. The animals in both the experimental and control groups received no further treatment. They were maintained at a temperature of 70° F. with a diet of Purina Fox Chow and water *ad libitum*.

Virgin and castrate mice were sacrificed at intervals ranging from 15 days to 26 months of age

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[1] G. W. Woolley, unpublished data.

and were immediately examined at autopsy. No attempt was made to sacrifice the animals at any particular stage of the estrous cycle. In each case the genital tract, the adrenals, pituitary, and submaxillary glands, etc. (and the ovaries in the intact virgins), were removed and fixed in modified Tellyesniczky's fluid (115 ml. of 95 per cent alcohol, 35.7 ml. of distilled water, 15 ml. of formalin, and 7.5 ml. of glacial acetic acid). Tissue specimens were imbedded in paraffin at 56° C. Sections were cut at 8 μ and were stained with Mayer's hematoxylin and aqueous eosin.

Criteria used for evidence of steroid activity.—In addition to the histopathological studies described below, sections of the uterus, vagina, and submaxillary glands were examined for evidence of response to steroid activity. In the uterus, the morphological criteria defined by Hooker (9) were used to identify the response to estrogenic and progesterone-like substances. According to Hooker, under estrogenic influence mitotic figures are seen in the epithelium, the epithelial cells increase in height, and their cytoplasm becomes granular. The endometrial stroma becomes edematous and infiltrated with polymorphonuclear leukocytes, and the stromal nuclei become fusiform and pyknotic. Grossly, the organ is hyperemic and distended. Under progesterone influence the epithelial cells are slightly taller than those of the castrate uterus, and their cytoplasm is clear. In the stroma the nuclei enlarge and become rounded, vesicular, and granular, with a prominent nucleolus. There is no edema or leukocytic infiltration. Under the influence of both hormones both types of change are seen concurrently. It should be noted here that, according to Hooker and Forbes who used special technics, desoxycorticosterone and testosterone administered systemically may produce endometrial nuclear changes indistinguishable from those produced by progesterone (10). Prolonged estrogenic stimulation of the uterus results in endometrial fibrosis and hyalinization—changes which are also phenomena of aging (16). Androgenic stimulation is manifested in a thick, fibrous stroma and in endometrial nuclei intermediate between the castrate and progesterone-like conditions.

The vagina was examined for evidence of cyclic stimulation. Specific hormonal effects were judged by the criteria used by Atkinson and Kamell in an earlier study (1). The action of estrogen alone is indicated by the proliferation of the vaginal epithelium with subsequent cornification of the superficial layers. On the other hand, proliferation followed by mucification of the superficial layers is evidence of progesterone activity either concurrent with or following estrogen stimulation. Mucification may also be the result of androgenic stimulation (12).[1]

The submaxillary gland reflects hormone stimulation grossly and microscopically. Under estrogen influence the gland is small and dark red grossly; microscopically, the terminal tubules are lined by low columnar cells with eosinophilic cytoplasm and vesicular nuclei in a central or apical position. Androgenic influence makes the gland large and pale, while in the terminal tubules the epithelial cells are tall columnar, with decreased cytoplasmic eosinophilia and with flattened nuclei in a very basal position (5, 13).

OBSERVATIONS

Gross Observations on the Uteri

Virgin Females

Grossly, the uteri of the virgin mice remained small in both F_1 groups until about 2 months of age, when post-puberal enlargement took place. Adult size and appearance were attained by 2 months in the DBA female × CE male series and by 4 months in the reciprocal group. The size of the organ in both groups remained within normal limits until 7–8 months of age. At this time the uterine horns began to increase in diameter. In the older age groups (15 months and older), a size up to 2–3 times that seen in early sexual maturity was attained. In most cases the horns were uniformly hypertrophied. In some, however, the enlargement was nodular in character. The hypertrophy and vascularity persisted until 26 months of age (the oldest animal in the series).

Gonadectomized Females

In the gonadectomized mice the uterus remained small until 5 months of age. By 6 months however, although the uterus was still small, there was evidence of beginning stimulation in the increased blood supply. By the eighth month, the uterus had enlarged to equal the size of the 8-month organ of the intact hybrid. This increase in size appeared after the development of adrenal cortical carcinoma. Adult size of the uterus persisted until 24 months (the oldest animal used), but the very marked hypertrophy seen in the intact virgin mice was not found.

Microscopic Observations on the Uteri Correlated with Adrenal Cortical Changes and with the Vaginal "Cycle"

Virgin DBA Female×CE Male F_1 Hybrids

One to 8 months.—The uteri of 1-month-old animals were small and undeveloped. In the two animals studied, there was no evidence of estrogenic stimulation of the uterus. Both lacked stromal

edema or hyperemia, and both had very few uterine glands. Clear evidence of estrogenic effect was seen at the age of 2 months. The 2-month uteri were adult in size. Several glands were present, and the surface (lumenal) and glandular epithelia showed estrogenic change (tall columnar cells, cytoplasmic granularity, folding, mitoses). The stroma showed mixed effects—round, vesicular, granular nuclei (i.e., progesterone-like changes) in the presence of edema and leukocytic infiltration (estrogenic changes). This was not unexpected, since, as mentioned above, no attempt was made to obtain specimens at a definite stage in the estrous cycle.

At from 3 to 7 months the uteri of this series showed increasing steroid sex hormone stimulation with morphological features which correspond more or less well to the observed vaginal estrous cycle. There was stratification of surface epithelium, an increase in the number of glands, and notable stromal stimulation by estrogen, progesterone, desoxycorticosterone, or testosterone. The myometria showed thickening, hyperemia, and edema (Fig. 1). At 6 months there was endometrial fibrosis associated with stromal nuclei intermediate between the fusiform and vesicular types. The vagina showed the metestrous morphology (cornified layer being delaminated, WBC in epithelial layers). From 8 months on, the uteri were all much increased in diameter. The 8-month uterus (in late estrus from the vaginal picture) was the first to show distinct pathologic change. Here, associated with a stroma showing a progesterone-like reaction there was glandular hyperplasia of both cystic and adenomatous types. The glands were very numerous, small, and closely spaced. The uterine lumen was extremely wide, and there were deep foldings of the surface epithelium. Adenomatous change of the uterine gland, defined by Hertig and Sommers (8) as an "outpouching and pinching off" resulting in groups of small, close-packed glands, was seen in several areas where the glandular epithelium was so stratified and folded that within a single basement membrane there were several minute lumina instead of one (Fig. 2). The absence of fine connective tissue and of a basement membrane in the interior of these formations suggests the hyperplasia of a single glandular element rather than the confluence of several. Cystic glandular hyperplasia was seen in one or two glands that had undergone tenfold enlargement, with flattening and patchy stratification of the epithelium. The cystic glands contained small amounts of lightly stained eosinophilic material.

Nine months and beyond.—In the 9-month uterus (obtained at estrus), stromal nuclei showing a progesterone-like reaction were accompanied by endometrial edema. The surface epithelium showed estrogenic change with some stratification. In addition, adenomyosis (internal endometriosis), i.e., invasion of the myometrium by glands and endometrial stroma, appeared for the first time. This lesion was also seen in the 10-month uterus (vaginal diestrus) (Fig. 3). Stromal nuclei again showed a reaction of the progesterone-like type. Leukocytic infiltration was apparent, but the epithelium exhibited no estrogenic changes. At three points in the circular muscle layer endometrial glands and stroma could be seen penetrating through, even reaching into, the longitudinal muscle layer. The invasive glands and stroma were not structurally different from the rest of the endometrium. In one area where the invading gland was sectioned longitudinally, a branch of the somewhat distended lumen could be followed in successive sections out into the myometrium. The myometrium was thick, hyperemic, and edematous, whereas in the normal diestrous female, the uterine wall is "collapsed and anemic" (18).

An 11-month uterus with a proestrous vagina and the 12-month uterus with diestrous vagina exhibited stromal nuclei with a progesterone-like reaction and mild, cystic glandular hyperplasia. In the 12-month uterus intense myometrial hyperemia was present, despite the diestrous phase of the cycle. At 13 months, the uterus (diestrous vagina) showed the same type of myometrium. Several foci of ectopic endometrium were noted in both muscle layers. Cystic glands could be seen in the endometrium proper and in the ectopic foci. Uteri from 14 through 17 months all had vesicular stromal nuclei and cystic glandular changes, as well as adenomyosis. The myometria of all were edematous, the 15-month uterus being distinguished by the presence of muscular hyperplasia. Another 15-month specimen displayed marked stromal edema, myometrial hyperplasia, cystic and adenomatous hyperplasia, with adenomyosis. Similar changes were found in the 18-month uterus. The 19-month organ was very large and was noteworthy as the only specimen in this virgin series which showed no nuclei with a progesterone-like change in the stroma. Observation of vaginal sections revealed an estrous condition. The myometrium was hyperplastic. The endometrial changes were the most extreme thus far observed. These changes included conspicuous stromal edema and great increase in the number of epithelial elements. Mixed with the numerous minute glands there were greatly distended glands and some showing adenomatous change. This specimen (Fig. 4) closely resembled

the so-called "Swiss-cheese endometrium" of human females (21). Changes as extreme as the above were also observed in the 20-month and in one of the 22-month uteri. The remaining 22-month and the 23- and 25-month uteri showed less marked pathologic change than those immediately preceding chronologically, but there was no indication in the accessory reproductive organs of decreased steroid hormone stimulation, even in these aged animals.

Virgin CE Female×DBA Male F_1 Hybrids

There were no important differences between the uteri of this and the reciprocal series of virgin hybrids. Although the 1- and 2-month animals showed vaginal cyclic activity, the uteri were small in size. The epithelium of the 2-month specimen had clear-cut evidence of estrogenic stimulation. Adult size was not reached until 4 months. From 5 to 7 months there was progressive increase in uterine size and glandular development, and at 7 months adenomyosis appeared. Cystic glandular hyperplasia first occurred at 8 months and adenomatous hyperplasia at 11 months. Large size, stroma showing a progesterone-like reaction, adenomyosis, and glandular hyperplasia persisted without diminution up to the age of 26 months. The uterus of a 22-month animal manifested perhaps the most extreme cystic hyperplasia of the series, in the presence of a lutein cyst of the ovary. From 23 to 26 months of age there was thickening and fibrosis of the myometrium. The 23-month specimen had overgrowth of collagenous connective tissue around stromal blood vessels.

Gonadectomized DBA Female×CE Male F_1 Hybrids

Changes due to progesterone-like stimulation (testosterone or desoxycorticosterone) appeared earlier in these gonadectomized animals than did estrogenic changes. In a 15-day animal, the uterus was small with thin muscle layers and presented the castrate appearance. No evidence of either estrogenic or progesterone stimulation could be found in the 1-month uterus. The 4-, 5-, and 6-month uteri had the castrate morphology, but the nuclei in the endometrial stroma were round and vesicular (Fig. 5). By 7 months the uterus began to show some enlargement, and, although there was as yet no neoplastic change in the hyperplastic nodules of the adrenal cortex, the vagina was in proestrus. (The vaginas of the younger animals in this series had all been unstimulated.) At this stage of development (7 months) the muscle was thin, stromal nuclei vesicular, and lumen wide. In addition to vaginal activity, other evidence of estrogenic effect which may have contributed to the uterine enlargement were the presence of many small uterine glands, tall granular epithelium, epithelial folding with some stratification, and small cysts.

Definite pathologic change was first seen at 8 months. It is worth emphasizing that this is the same age at which glandular hyperplasia first developed in the intact virgins. The vaginal phase was late estrus. The uterus was large and had a well developed myometrium. The nuclei of the endometrial stroma were vesicular, but leukocytic infiltration of the stroma indicated some estrogenic influence. There was also considerable collagenous connective tissue in the stroma, a condition consistently found up to 21 months of age. The epithelium reflected predominantly estrogenic stimulation. The lumen was wide. The surface and glandular epithelium was very tall and granular. Although the glands were relatively few in number, cystic and adenomatous changes were seen.

The uteri of the 9-month animals, while similar to those at 8 months, had thicker muscle layers. These 9-month animals were the first to develop adrenal cortical carcinoma. All animals from 10 months on had bilateral adrenal cortical carcinomas. The 10- and 12-month uteri were similar to the above, with more pronounced cystic hyperplasia. The 14-, 16-, and 17-month uteri continued to show the same changes—stromal fibrosis, cystic and adenomatous hyperplasia, with the added feature of adenomyosis (Fig. 6). It is noteworthy that adenomyosis appeared first in these ovariectomized animals at 14 months while it was first demonstrable at 9 months in the corresponding virgin series.

Many of the gonadectomized animals showed pituitary basophile tumors after the age of 17 months (4). From 17 to 21 months the uteri showed, in addition to all the above changes, a peculiar increase in collagenous connective tissue around small vessels and lymphatics in the endometrium (Fig. 8). An increase in collagenous fibers, both diffusely distributed and localized about small vessels was noted in the myometrium. Finally, in two cases (18- and 19-month animals), a displacement of the nuclei of surface and glandular epithelium toward the apices of the cells was observed (Fig. 9). This phenomenon has been reported previously in the uteri of aged CE strain mice (26). There was no evidence in the uteri to indicate decreased hormonal activity with advancing age.

Gonadectomized CE Female×DBA Male F_1 Hybrids

Reciprocal differences in the two series of gonadectomized females were not striking. As in the reciprocal series of castrate females, evidence

of some hormonal influence other than estrogenic was seen before estrogenic stimulation occurred. There was no uterine or vaginal enlargement in the 15-day and 1-month animals. The 4-month uterus showed the castrate picture. The uterus at 5 months had epithelium of the castrate type, but the stromal nuclei were vesicular. At 6 months, such vesicular stromal nuclei were accompanied by estrogen-stimulated epithelium, and the vagina had four to five epithelial layers with some superficial mucification. This 6-month animal exhibited an adrenal neoplasm. From 7 months on, "estrous" vagina was associated with an adult-sized uterus which had stromal fibrosis—a change found consistently throughout the series until 24 months of age. In the 7- and 8-month specimens, high degrees of estrogenic stimulation of the epithelial elements and edema and leukocytic infiltration of the stroma could be seen. Cystic glandular hyperplasia first made its appearance at 9 months, together with adenomatous hyperplasia. It will be remembered that these lesions first occurred at 8 months of age in the intact hybrids of this cross. All animals from 9 to 24 months had adrenal cortical carcinomas which were usually bilateral. From 10 to 15 months, high estrogen levels are expressed by the stromal and epithelial morphology. In the 12-month uterus some hyaline change could be observed in the connective tissue of the stroma. This might indicate prolonged, high-level estrogenic stimulation, according to Loeb *et al.* (16). The 15-month uterus had the most advanced cystic hyperplasia.

From 14 to 24 months, many of the animals had basophile adenomas of the anterior pituitary (4). The myometria of the uteri of these animals showed increasing edema, smooth-muscle hyperplasia, and fibrosis, more pronounced with advancing age. Vesicular stromal nuclei and fibrosis of the endometrium persisted until the end of the series. Increased amounts of perivascular and perilymphatic collagenous connective tissue were first seen at 18 months and were present in about half of the 18- to 24-month uteri. Surface and glandular epithelium indicated undiminished estrogenic stimulation, even in the oldest animals. Adenomyosis was present in the 16-, 17-, 20-, 22-, 23-, and 24-month specimens (Fig. 7). (Note that this change first occurred at 7 months in the corresponding intact females.) Cystic hyperplasia and adenomatous hyperplasia were also seen up to 23 months of age. The 21-, 23-, and 24-month animals demonstrated the peculiar migration of epithelial nuclei from the basal to the apical portions of the cells, a change which first occurred at 18 months in the uteri of the reciprocal castrate series.

Evidence of Endocrine Dysfunction in Structures Other than the Uterus

The uterine changes in both control and gonadectomized female hybrids showed clear evidence of an altered internal environment with respect to endocrine secretions, an alteration detectable even in old animals. The other accessory reproductive structures also offered such evidence as did the endocrine glands. Details of vaginal, submaxillary, adrenal, and ovarian abnormalities will be reported later, and those of pituitary and mammary glands have already been described (4), so that only a few general remarks bearing on the endocrine factors involved in the genesis of the uterine lesions need be made here. The vagina, as has been indicated above, responds to steroid stimulation in both intact and castrate females. Following adrenal hyperplasia in the castrates, the vagina shows evidence of hormonal stimulation, but, unlike the uterus, it usually reverts to the castrate morphology in the older animals. The submaxillary gland, by its differential response, serves as an indicator of estrogen and androgen activity. In the control females, the submaxillary is predominantly female in type, with signs of low androgen activity appearing soon after sexual maturity. In the castrates, at a given age, androgenic activity is more pronounced and becomes quite extreme in some of the older animals. The androgenic effects follow the development of adrenal hyperplasia and precede the appearance of marked steroid stimulation in either the uterus or vagina.

In regard to the endocrine glands themselves, the ovary in the intact hybrids undergoes cyclic changes, paralleled by the vaginal cycle, for several months following sexual maturity. In older animals, degenerative changes appear—decreasing numbers of follicles, increasing amounts of lipochrome pigment, increasing numbers of hyalinized corpora lutea, calcification, and an occasional cyst in older mice. In spite of the progressive morphological regression of the ovaries, there is, as has been shown, no uterine evidence of decreased estrogenic activity with advancing age. The adrenal changes in the castrates are comparable to those described in one of the parent strains (CE) by Woolley and Little (24, 26). Changes in the adrenals of intact animals will be discussed in a forthcoming report. Most pertinent to the present investigation is the observation that in the castrate hybrids adrenal cortical hyperplasia *always* precedes morphological evidence of growth and marked steroid stimulation of the accessory reproductive structures. Previous studies have suggested that the basophile nodules of the pituitary appearing in the older gonadectomized animals

may have an endocrine function, an inference made on the basis of the "extreme alveolar development of the mammary glands" in these animals, a change which accompanies the pituitary tumors and never occurs in their absence (4). There is no clear-cut evidence in the uterus of such a *direct* anterior pituitary effect.

DISCUSSION

The results demonstrate the occurrence in hybrid mice of uterine lesions in the presence of endocrine secretions which differ either qualitatively or quantitatively from the normal. Smith has reported the prevalence of such lesions in gonadectomized mice bearing adrenal tumors (17). The present studies show that pathological endometrial changes may develop in intact animals without adrenal cortical carcinoma, as well as in the castrates which have cortical neoplasms. Adrenal cortical neoplasia per se is thus seen not to be the only or the essential factor in the pathogenesis of the endometrial abnormalities. Rather, the adrenal cortical tumors appear to be but one manifestation of a generalized alteration of the endocrine "milieu intérieur." Moreover, the intact female need not be exempt from suspicion of adrenal dysfunction merely because of the absence of histopathologic evidence of carcinoma.

We shall attempt to show how endocrine factors operate in the development of the uterine changes and, in so doing, to throw some light on the nature of the altered endocrine status of these animals. Chart 1 is a diagrammatic representation of the occurrence of some of these uterine changes.

Cystic Glandular Hyperplasia

The most prominent uterine change observed is cystic glandular hyperplasia. There is a great deal of experimental and clinical evidence pointing to *hyperestrinism* as the most important factor in the development of this condition (21). A simple excess of estrogenic hormone is not the essential agent, but, rather, continuity of estrogenic stimulation without the periods of rest provided in the normal menstrual or estrous cycle. The experiments of Burch, Zondek, and others, cited in Taylor's excellent review (21), show that the typical picture of cystic hyperplasia can be produced by estrogen injection. But the work of Lipschütz, in which partial ovariectomy in guinea pigs produced ovari-

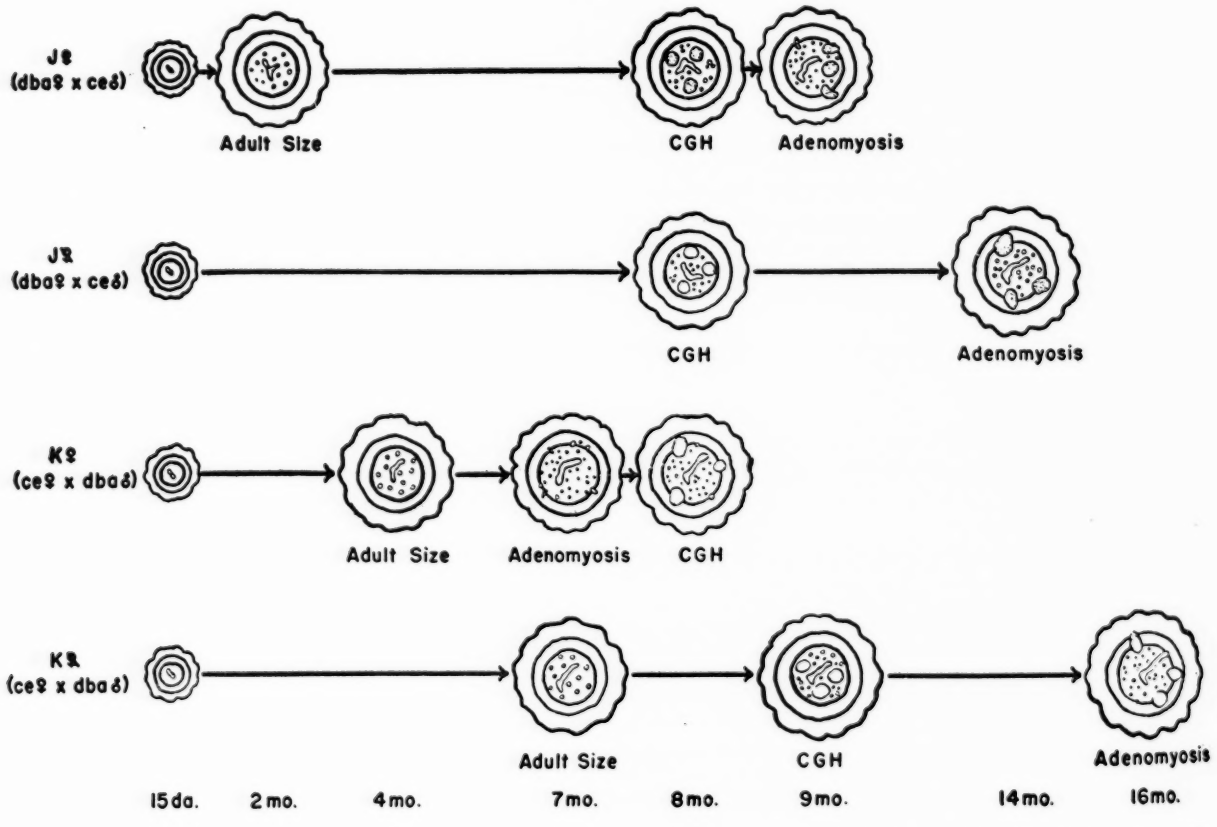

Chart 1.—Diagrammatic representation of cross sections of uteri at critical periods during life. The four groups of females—virgin DBA × CE F_1, gonadectomized DBA × CE F_1, virgin CE × DBA F_1, and gonadectomized CE × DBA F_1— are compared.

an hypertrophy with absent or incomplete corpus luteum formation and consequent endometrial hyperplasia, clearly demonstrates that the lesion is more nearly related to disturbed rather than simple excessive ovarian function (14, 15). Clinical evidence of hyperestrinism in association with endometrial hyperplasia has been shown by several workers (21) in the form of increased excretion of estrogen in the urine.

In these experimental animals, intact and gonadectomized, the presence of estrogen is indicated by vaginal cornification, by growth of the uterus and mammary glands, and by clear-cut estrogenic changes in the cytology of the uterine epithelium and stroma. There is also evidence, some of it inferential, that estrogen secretion in our animals is both *excessive* and *prolonged*. Dorfman and Gardner, working with NH strain female mice bearing adrenal tumors (3), demonstrated a fourfold increase of urinary estrogen excretion in their animals. Such determinations have not been made on the DBA × CE hybrids, but we may assume a similar hyperestrinism on the basis of several histopathologic findings. First, the mice show uterine overgrowth, most marked in the intact females, and myometrial hyperemia and edema frequently occur in the face of a diestrous vaginal condition. Second, many of the uteri show extreme epithelial folding, and, in some instances, stratification. Third, endometrial fibrosis (and hyaline change in some cases) is constantly present in intact and gonadectomized females after the age of 8 months. This phenomenon has been produced in mice by the administration of estrogen (16). Burack *et al.* (2) associate deposition of collagenous connective tissue in the endometrium, in the myometrium, and about the intra-uterine arteries, as well as increase in uterine size, with long-continued, unopposed activity of estrogen in rats. This conclusion was based on the finding that the above changes are more extensive in virgin than in breeding animals. We have made similar observations (unpublished) on DBA and CE female mice. In these animals, the parent strains of our experimental hybrids, changes like those found in the hybrids (and in Burack's rats), are seen more often and in more severe forms in gonadectomized females (with adrenal cortical hyperplasia or carcinoma) and in virgin females (without extensive adrenal pathologic change) than in the animals that have borne several litters. Fourth, in support of the idea that the hyperestrinism in our hybrids is prolonged as well as excessive, it should be noted again that hyperplastic and other uterine changes persist into old age.

There is ample evidence that the source of estrogenic hormone in gonadectomized mice with adrenal cortical hyperplasia or neoplasms is the abnormal adrenal cortex (4, 6, 17, 23, 25, 26, 28). That adrenal cortical estrogen is probably the factor responsible for the excessive endometrial stimulation which results in hyperplasia in the intact as well as the castrate animal is strongly suggested by the onset of the endometrial hyperplasia at very nearly the same age in both intact and castrate females. The ovary is apparently not necessary as an estrogen source for the development of the lesion.

Adenomatous Hyperplasia

The relation of adenomatous hyperplasia of the endometrium to hyperestrinism is not so clearly indicated by experimental work. Hertig and Sommers (8) question whether the lesion is due to excessive estrogen or to abnormal pituitary-ovarian or pituitary-adrenal relationships. In a 16-month-old diethylstilbestrol-treated CE female reported by Woolley and Little (27), adenomatous hyperplasia was seen in association with beginning adenocarcinoma of the endometrium, which suggests that this type of hyperplasia may be precancerous in the mouse as it apparently is in the human (8). It is noteworthy that in none of the DBA × CE hybrid uteri were there changes indicative of carcinomatous transformation in foci of adenomatous hyperplasia.

Adenomyosis

Adenomyosis appears to be related to estrogenic stimulation. Taylor states that in women the lesion is found only when the ovaries are active (21). Lipschütz reported adenomyosis in his partially ovariectomized guinea pigs which showed cystic hyperplasia (15). In our experimental series, adenomyosis appeared later in the castrate than in the virgin uteri. Why this should be is not entirely clear, unless it is an expression of a quantitative difference in estrogenic activity in the two groups.

Hypothetical Endocrine Mechanisms in the Production of Endometrial Lesions

The principal uterine lesions in DBA × CE hybrid mice appear to be related, at least in part, to prolonged high levels of estrogen, of which the main source is presumably the adrenal cortex, whether morphologically neoplastic or not. However, as has been indicated, there is also evidence of other hormonal activity. The stromal nuclei of the uterus show changes which may be progesterone-like reactions (9) or secondary to testosterone or desoxycorticosterone stimulation (10). Endometrial fibrosis which is so constant a feature here may also be produced, in rats at least, by ad-

ministration of testosterone (12). Vaginal mucification, seen in our old castrate hybrids, may be an androgenic effect (12),[1] a fact which is emphasized by the prominent androgenic changes in the submaxillary glands of these animals. What role androgens, adrenal cortical steroids, and progesterone may play in association with estrogens in the etiology of endometrial hyperplasia and adenomyosis is obscure. However, in gonadectomized CE females, in which adrenal cortical tumor formation is suppressed by implantation of pellets of potent estrogenic hormones, cystic glandular hyperplasia has not been seen and adenomyosis and adenomatous hyperplasia only rarely.[1] The inference is that in such CE animals and perhaps in our hybrids a mixture of several steroids acting synergistically and in improper balance, rather than a single predominant hormone, is required for the production of the uterine picture seen in the present series. How much of the pathologic change is due to intrinsic uterine tissue differences and how much to altered endocrine environment are questions as yet unanswered.

Pituitary-adrenal and pituitary-ovarian relationships.—The uterine cytology is similar in the castrate and intact females. This implies a rough similarity in the pattern of steroid secretion in the two groups, with more androgenic predominance in the older castrates, as seen in the submaxillary morphology. Because the suspected common endocrine denominators in the two groups are the adrenal cortex and the pituitary, since comparable uterine lesions (cystic hyperplasia) appear at approximately the same age in castrate and intact alike, we may assume a corresponding similarity in hypophyseal and adrenal cortical dysfunction on a *physiologic* level in the two groups. What is the origin of the dysfunction? Genetic factors must play a part, in view of the incidence in the parent strains DBA and CE, of very similar endometrial hyperplastic phenomena[1] which as in their hybrid offspring are only one manifestation of widespread endocrine abnormality. Closer to this discussion is the hypothesis of Dickie and Woolley (4) who postulate the anterior pituitary as the origin of the stimulus for the adrenal cortical change in DBA × CE and other hybrids. The suggested mechanism of the pituitary-adrenal relationship is that in the castrate, which has both adrenal and pituitary tumors, the fundamental change may occur first in the pituitary, even though morphological pituitary change does not develop until after structural change of the adrenal. The pituitary may thus react on the adrenal, the resultant adrenal secretions then reacting on the pituitary in turn, so that structural hypophyseal changes are not apparent until after the adrenal cortex becomes neoplastic (4).

We must go one step further with this hypothesis to explain the uterine evidence in the intact virgins of endocrine dysfunction, so similar to that seen in the castrate animals, despite the absence in the virgins of pituitary or adrenal tumors. The assumption is made that in the intact female hybrid the ovarian steroids inhibit the action of the anterior pituitary sufficiently to prevent the extreme hypophyseal activity (and basophile change) seen in the castrate, but not sufficiently to prevent physiological over-stimulation of the adrenal cortex which, in response, secretes an excess of steroids with consequent uterine maldevelopment, without itself becoming carcinomatous. We do not attempt to answer definitively Kepler's question as to whether the adrenal cortex or the pituitary basophile cells are primarily the cause of this experimental type of Cushing's syndrome (11), but we wish to point out that there is suggestive evidence from our uterine studies that the pituitary and adrenal may both be *physiologically* hyperactive without showing marked *structural* change.

The nature of the pituitary and adrenal hormones.—The final step in our hypothesis concerning the origin of the abnormal endocrine conditions in these hybrids has already been indicated by Dickie and Woolley—"the substance or substances . . . from the pituitary and adrenal glands seem to be of a gonadotrophic and gonadal nature respectively" (4). That the adrenal cortical steroids are gonadal in nature has been shown at length in the foregoing observations. The gonadotrophic potentialities of the pituitary in this connection may be inferred from several pieces of evidence. Frantz *et al.* (7) have shown that gonadotrophic hormones injected into adrenalectomized NH female mice fail to produce an ovarian response in terms of estrogenic effect on the vaginal smear. However, these workers were able to demonstrate vaginal evidence of estrogenic hormone secretion following gonadotrophin administration to gonadectomized NH females *with adrenal cortical tumors.*

Evidence from this laboratory that the pituitary stimulus to the adrenal may be gonadotrophic is afforded by three findings. (*a*) Ovarian steroids, known to inhibit gonadotrophic activity, appear to reduce the atypical adrenocorticotrophic effects by preventing the development of adrenal cortical carcinoma in the intact females of the series presented here. (*b*) Potent estrogenic and androgenic steroids, both of which inhibit gonadotrophic action, have been shown to suppress the development of adrenal cortical neoplasms in CE gonadectomized females (22, 27). (*c*) Unpublished experi-

ments[1] suggest that, although ACTH is not effective in stimulating hyperplastic DBA strain adrenal cortices to neoplasm-formation, FSH (and to a lesser extent LH) may be effective. (Intense study of these data has not yet been completed.)

These experimental results suggest that pituitary gonadotrophins acting on the adrenal cortex are the origin of hyperplastic changes in the uteri of DBA × CE hybrid mice. This idea is in accord with the speculations of Sommers *et al.* (19) and of Speert (20) who indicate that pituitary-adrenal factors may operate in the pathogenesis of endometrial hyperplasia and carcinoma.

SUMMARY

The pathogenesis of the uterine lesions in intact virgin and gonadectomized hybrid mice of the F_1 reciprocal crosses between the DBA and CE strains was studied in animals sacrificed from 15 days to 26 months of age. Cystic hyperplasia of the endometrial glands appeared in both the intact and gonadectomized mice at about 8 months of age. The cystic changes were frequently accompanied by adenomatous hyperplasia of the glandular epithelium. In many of the intact animals these conditions were seen concurrently with invasion of the myometrium by the endometrial glands and surrounding stroma (adenomyosis or myometrial endometriosis). In the gonadectomized group, however, adenomyosis occurred later—not before 14 months of age.

Adrenal cortical neoplasms developed in gonadectomized mice by 6–9 months of age. However, the occurrence of uterine lesions in the intact animals indicates that the morphologic changes in the adrenals of the castrates are not necessary per se to the development of the endometrial lesions.

The endocrine status of the intact and gonadectomized mice with respect to the activity of the steroid sex hormones was assayed on the basis of the well established cytological changes which are found in the uterus, vagina, and submaxillary glands. Microscopic study of these organs indicated the presence of an endocrine imbalance first manifested at about 6–8 months and persisting to extreme old age, in both the intact and gonadectomized animals. This imbalance was characterized most notably by the continuous, noncyclic, prolonged, and excessive production of estrogen. Since the phenomena are essentially the same in both the intact and gonadectomized mice, the adrenal cortex is presumed to be the source of the aberrant estrogenic hormone.

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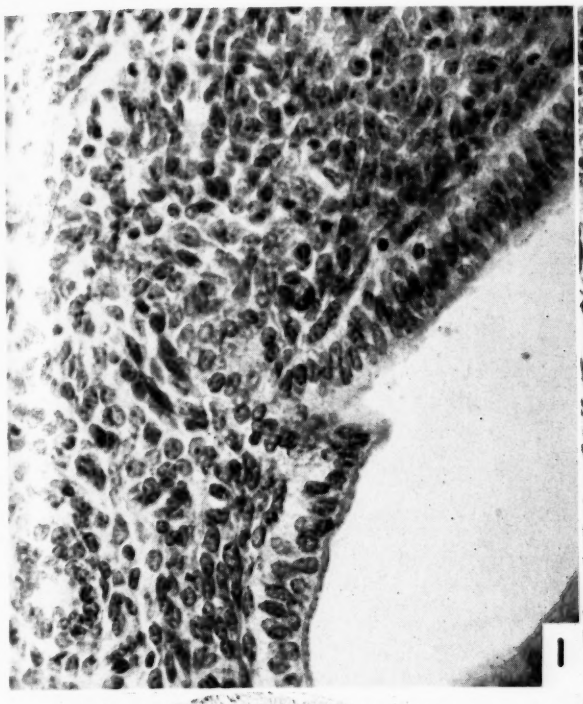
Fig. 1.—Section of endometrial stroma and epithelium of a 4-month virgin DBA ♀ × CE ♂ F_1 female WK913. ×350.

Fig. 2.—Beginning of cystic and adenomatous changes in 8-month gonadectomized DBA × CE F_1 female WK140. Note several lumina within one gland. ×350.

Fig. 3.—Adenomyosis is present in the cross-sections of the uterus of a 10-month virgin DBA × CE F_1 female WK414. ×25.

Fig. 4.—Advanced adenomyosis and cystic glandular hyperplasia make the cross-section of the uterus of this 19-month virgin DBA × CE F_1 female WK753 somewhat comparable to the "Swiss cheese endometrium" found in humans. ×10.

Fig. 5.—Endometrial stroma and epithelium of a 4-month gonadectomized DBA × CE F_1 female WK32. Note nuclei of stroma are round and vesicular, while the uterus is castrate in size and structure. ×350.



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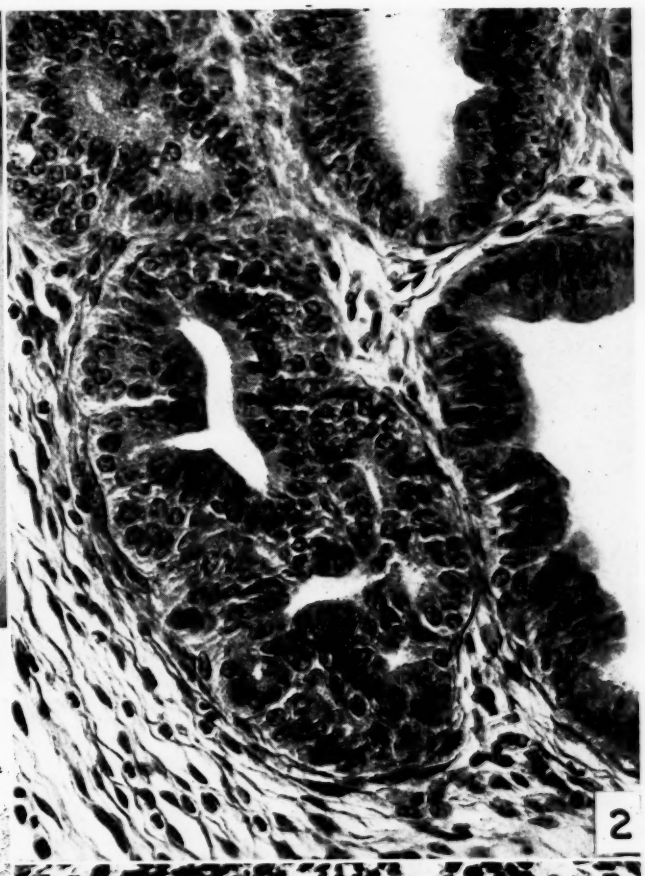

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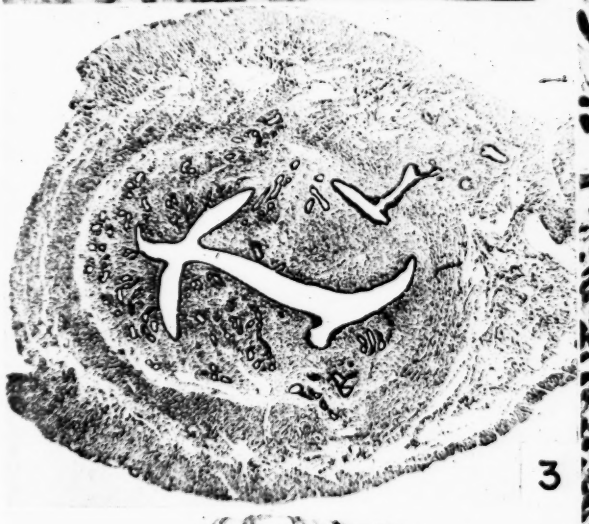

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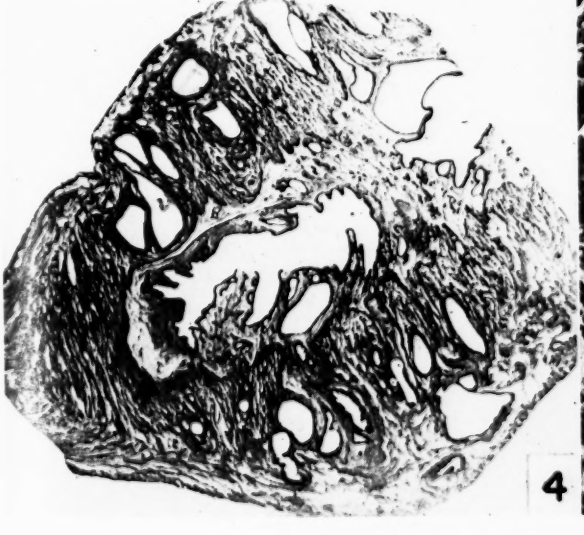

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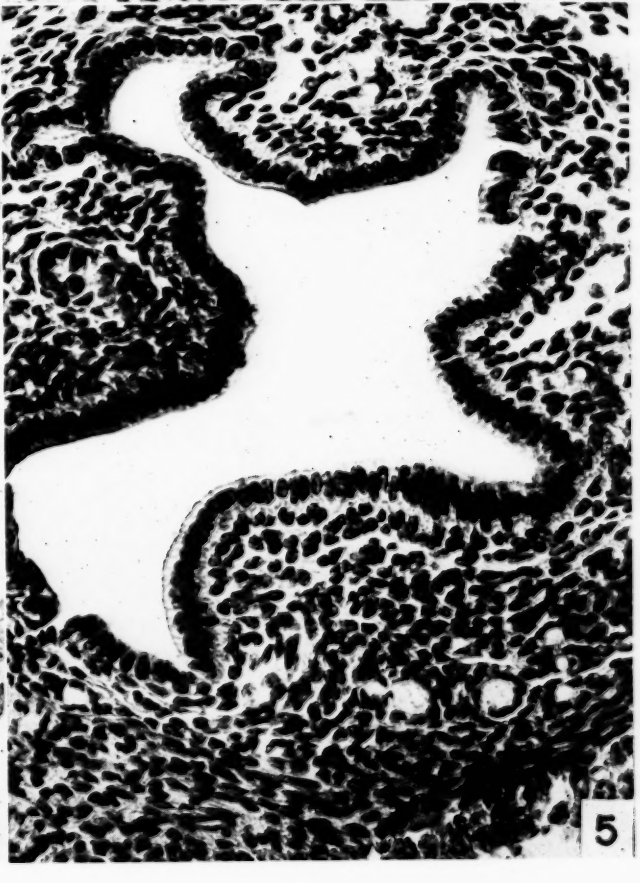

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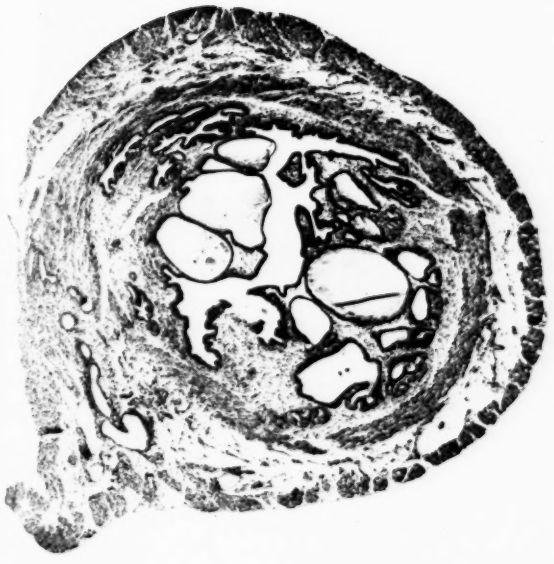
FIG. 6.—Adenomyosis as well as cystic hyperplasia can be seen in 14-month gonadectomized DBA × CE F_1 female WK328. ×25.

FIG. 7.—Adenomyosis and cystic hyperplasia found in the reciprocal group of females. This is a 17-month gonadectomized CE ♀ × DBA ♂ F_1 female WK397. ×25.

FIG. 8.—Perivascular connective tissue as seen in a virgin female of parent strain CE after 24 months. Same condition can be found in the reciprocal hybrid females. ×47.5.

FIG. 9.—Migration of nuclei of surface and glandular epithelium toward the lumen found in an 18-month gonadectomized DBA × CE F_1 female WK461. ×350.



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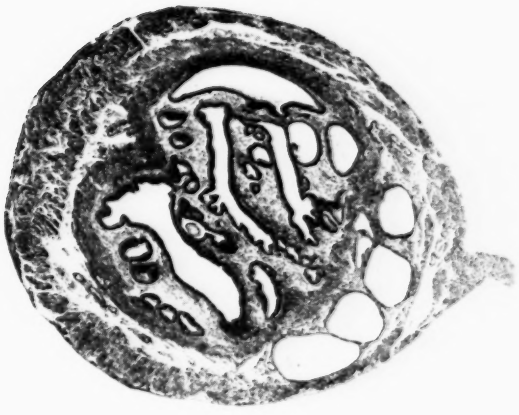

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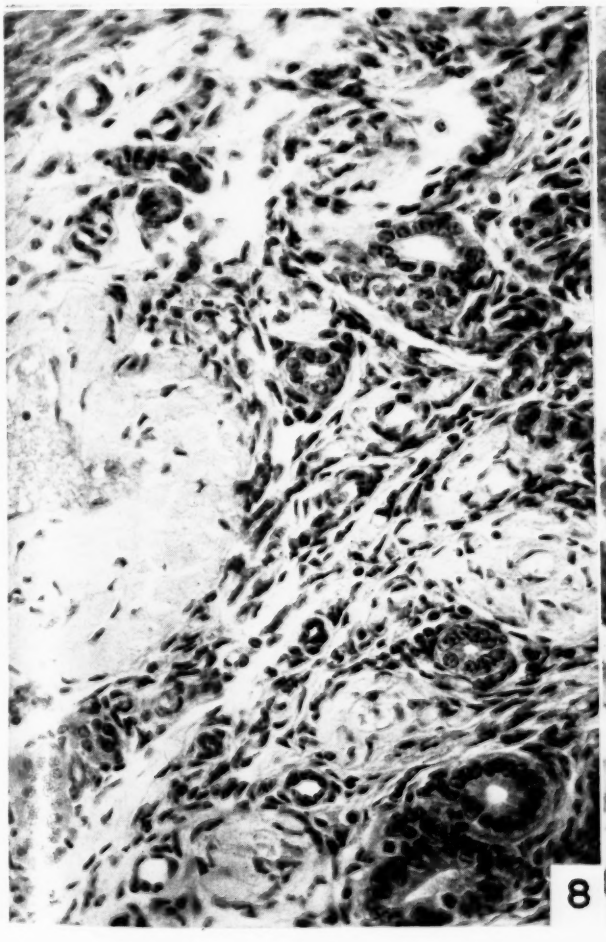

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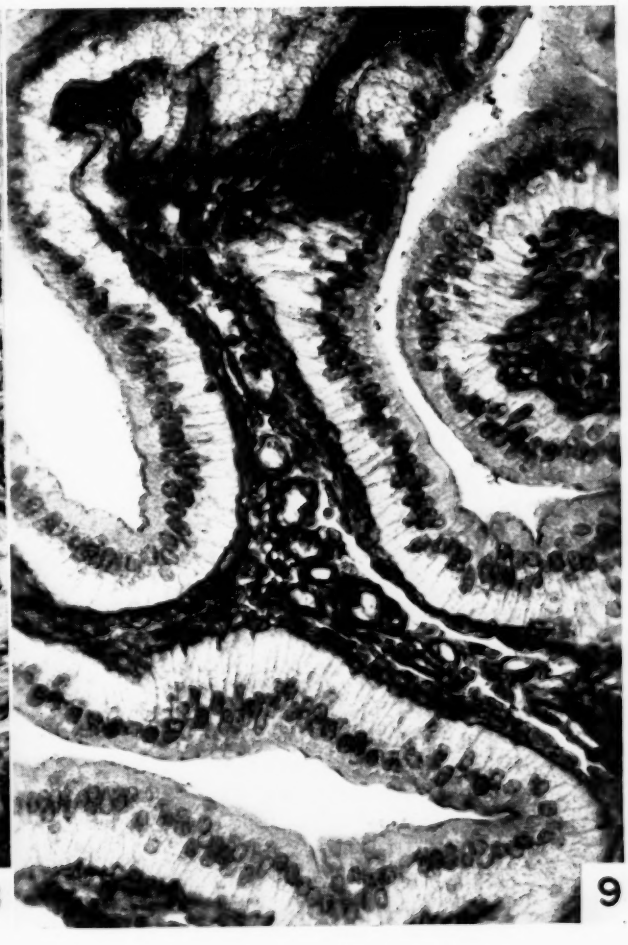

9

Carcinogenic Derivatives of Fluorene Containing Isotopic Nitrogen*

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Through the work of the last decade it has been definitely established that 2-acetylaminofluorene (AAF) is an active carcinogen, producing a variety of tumors in the animal body at numerous sites often far removed from the point of application (1, 10, 11). These characteristics of the compound make it useful for studying some of the fundamental factors of carcinogenesis.

A satisfactory method for its quantitative estimation in the animal body is necessary for such a study. A photometric method of estimation has been previously used, which involves hydrolysis of AAF to the free amine (AF), diazotization, and coupling with a known naphthol derivative to give a colored compound (9). By this method, however, only a 32 per cent recovery of AAF is obtained, indicating that in a very short time two-thirds of the material no longer has a diazotizable primary amino group (5).

Recently, preparation and investigation of the radioactive carcinogens, 2-acetylaminofluorene-9-C^{14} and 2-acetylominofluorene-ω-C^{14}, have greatly facilitated the metabolic studies of AAF. Since well over 90 per cent of the radioactivity may be accounted for in the animal body, these compounds have permitted a study of the pathways taken by the fluorene radical and the acetyl group of the compound (4, 8).

The fate of the nitrogen atom, however, is yet to be determined. As has been indicated before, a diazotizable amino group is necessary for AAF estimation by the photometric method. The low percentage recovery by this method indicates, therefore, either that the nitrogen atom, to a large extent, has been removed from the fluorene molecule or that the primary amino nitrogen has been rendered nondiazotizable, possibly by being converted to a secondary or tertiary amino group. This second possibility may involve the formation of a nitrogen ring compound similar to that prepared *in vitro* by the reaction of AF with pyruvic acid (6).

The importance of finding a method which would enable a decision to be reached between these two alternatives is evident. Synthesis of AAF with a labeled nitrogen atom which could be traced in the animal body was therefore undertaken. Since no radioactive form of nitrogen is available, we have employed the heavy isotope, N^{15}. The concentration of the isotopic nitrogen can be determined in various animal tissues by means of the mass spectrograph. By comparing the pathway of the 2-acetylaminofluorene-N^{15} with that of the radioactive compound, it will be possible to determine if and where in the animal body nitrogen is removed from the fluorene radical or where the nitrogen undergoes the metabolic change which renders it incapable of diazotization.

The usual method of preparation of AAF is the nitration of fluorene to 2-nitrofluorene (NF), reduction with Zn and 78 per cent ethyl alcohol to AF (2), and acetylation with acetic anhydride. Because of the high cost of N^{15} compounds, the ordinary methods of nitration which use 200–300 per cent excess concentrated nitric acid could not be employed. In addition, the N^{15} nitric acid is a dilute (approximately 2 M) solution, so it was necessary to work out a satisfactory method of eliminating the water and of using equal molar quantities of fluorene and $HN^{15}O_3$. This was accomplished by employing acetic anhydride as a solvent and dehydrating agent and concentrated H_2SO_4 as a dehydrating agent and catalyst. The yield based on nitric acid was 78.9 per cent, as compared to a calculated 21.4 per cent by previous methods (2).

Since the two intermediates, NF and AF, have also been shown to be carcinogenic (1, 3), samples of these were isolated for study.

A process was also worked out for a one-step reduction and acetylation of NF to AAF. This procedure conserves both time and reagents, although it does not substantially improve the yield as had been hoped.

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EXPERIMENTAL

2-Nitrofluorene-N^{15}.—Fluorene, 10.47 gm. (0.063 moles), was dissolved in acetic anhydride, 131.4 ml. (1.39 moles), which was sufficient to take care of the 1.35 moles of water in the nitric acid. The solution was heated to 55° C., and 26.47 ml. (0.063 moles) of 2.39 M HNO_3 containing 62 atom per cent N^{15} was added dropwise over a period of 1 hour during which the temperature was kept between 50°–60° C. Then, concentrated sulfuric acid, 5 ml. (0.08 moles), was added dropwise at 55° to react with the water formed and to catalyze the reaction. This precipitated the 2-nitrofluorene-N^{15}. The filtrate was removed by means of a filter-stick, and the yellow product washed by stirring with water and 1 per cent sodium acetate until neutral; the melting point was 154°; Kuhn (2) found 155°–156°; the yield was 78.9 per cent.

2-Aminofluorene-N^{15}.—A paste of 2-nitrofluorene-N^{15}, 3.3 gm. (0.015 moles), and 78 per cent ethyl alcohol, 110 ml., to which was added Zn dust, 33 gm., $CaCl_2$, 1.1 gm. (in 1.5 ml. of water), and charcoal, 1 gm., was heated to reflux with a microburner. Until the mixture began to reflux, the flask was shaken with a rotary motion to keep the contents well mixed. The mixture was boiled vigorously for 2 hours. Zn dust, 10 gm., was again added and refluxing continued for an additional 2 hours. The hot filtrate, which was removed by means of a filter-stick and illuminating gas pressure to avoid undue contact with air, was added to 600 ml. of water to precipitate the white product; the melting point was 126°; Kuhn (2) found 127.5°; the yield was 78.5 per cent.

2-Acetylaminofluorene-N^{15}. — 2-Aminofluorene-N^{15}, 5 gm. (0.027 moles), was dissolved in 20 ml. benzene, and heated to reflux. Acetic anhydride, 7 ml. (0.074 moles), was added dropwise and the mixture refluxed 30 minutes. The product precipitated on cooling and was recrystallized by dissolving in hot 95 per cent ethyl alcohol, adding water until turbid, filtering hot, and allowing the product to crystallize; the melting point was 191°; Poraĭ-Koshits and Nikiforova (7) reported 186°; Morris and Westfall (5) gave 194°; the yield was 90 per cent. As no extraneous nitric acid was used, the compound should contain 62 atom per cent of N^{15}.

2-Acetylaminofluorene directly from 2-nitrofluorene.—Glacial acetic acid, 250 ml. (0.023 moles), 2-nitrofluorene, 5 gm. (0.023 moles), Zn dust, 20 gm., and charcoal, 2 gm., were heated by means of a microburner to reflux with vigorous shaking. After hard refluxing for 2 hours, more Zn dust, 10 gm., was added and the mixture refluxed an additional 2 hours. Acetic anhydride, 10 ml. (0.077 moles), was added dropwise and the refluxing continued 1 more hour. The mixture was filtered hot and the product precipitated by addition of the filtrate to 700 ml. of water and recrystallized as described above; m.p., 190.5°; yield, 66 per cent.

SUMMARY

A synthesis is described which incorporates isotopic nitrogen (N^{15}) into the molecule of the carcinogen, 2-acetylaminofluorene. By mass-spectrographic analysis the hitherto unknown fate of the nitrogen during metabolism may be determined.

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Some Effects of Polysaccharide Preparations from *Serratia marcescens* and *Aerobacter aerogenes* on Cells in Tissue Culture*

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When certain polysaccharide fractions isolated from cultures of *Serratia marcescens* (6, 2), *Aerobacter aerogenes*, or other Gram-negative bacteria (3) are injected in large doses into mice, symptoms very similar to those of shock occur. The animals are prostrated, their muscular response is much lower than normal, they breathe with difficulty, and the body temperature drops. Similar symptoms are observed when these polysaccharides are injected into sarcomatous mice, but, in addition, zones of hemorrhage and necrosis appear in the tumor itself, sometimes resulting in the destruction of the neoplasm. Whether this necrosis occurs as a result of the direct action of the polysaccharide on the tumor cell or on the action of a complex formed *in vivo* after the polysaccharide injection, or results indirectly from a systemic disturbance, is not yet settled. Tissue culture technics may be helpful in solving it. It appears that the only observations along these lines are those of McConnell (4), who reported no damaging effect by a polysaccharide from *S. marcescens* (obtained from Dr. M. J. Shear) on cultures of Sarcoma 37 cells. Our own experiments bring out some facts which may be of interest.

MATERIALS AND METHODS

The cultures were carried out according to the hanging drop technic. The basic culture medium consisted of one drop of chicken embryo extract diluted 1:1 in Tyrode's solution and one drop of chicken plasma. The effect of the polysaccharide preparations was studied in two ways: (*a*) by direct inclusion in the nutrient at concentrations ranging from 3.3 to 800 μg/ml and (*b*) by permitting tissues to stand for 4 hours in solutions containing polysaccharide material, after which interval the cultures were transferred to the control nutrient. The cultured cells were tissue explants taken from 4-day chick embryo hearts (myocardial fibroblasts) or from Sarcoma 37 tumors in Swiss mice.

Most of the experiments were conducted with preparation A-2Y from *S. marcescens*, the remainder with preparation A-2 from *A. aerogenes*. The polysaccharides were obtained by the phenol-trichloroacetic acid method of Perrault and Shear (5) and after tryptic digestion were nondialysable and protein negative. The N content of A-2Y was 2.2 per cent, the P content 1.4 per cent, and reducing sugars (calculated as glucose) 54.2 per cent. The corresponding values for A-2 were 2.8, 1.2, and 57.9. A dose of 100 μg. of A-2Y was lethal in 96 hours for about 15 per cent of 8-week-old mice injected intraperitoneally and produced pronounced hemorrhage and necrosis of tumors in mice; 380 μg. of A-2 had a somewhat similar lethal effect in normal mice, and 100 μg. was highly lethal, hemorrhage-inducing, and tumor-necrotizing in tumor-bearing mice.

RESULTS

Action on normal chicken fibroblasts and on Sarcoma 37 cells.—A saline solution of A-2Y, introduced into the medium of cultures of normal chicken fibroblasts, at a final concentration of 20 μg/ml, had no effect on the cellular migration and areal increment of the tissue fragments. After 48 hours, the area in the controls with Tyrode's solution (50 cultures) was approximately the same as in the presence of polysaccharide (50 cultures). These experiments, repeated 3 times (300 cultures in all), confirmed the results of McConnell (4). When fragments of myocardium from a 4-day-old chick embryo were placed for 4 hours in contact with the polysaccharide solution at a concentration of 80 μg/ml in saline and were then grown in normal medium, they showed after 24 hours a much greater cell migration than the controls.

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After 48 hours the relative increase in diameter was greater by about one-half than in the controls where the tissue fragments had been in contact with the Tyrode's solution only (Table 1).

TABLE 1

RELATIVE INCREASE IN DIAMETER OF EXPLANTS OF NORMAL CHICKEN FIBROBLASTS IN SOLUTIONS OF POLYSACCHARIDE A-2Y

(50 cultures in each solution)

Hours	20 μg/ml nutrient	80 μg/ml, 4 hrs. contact, transf. to basic med.	Control Tyrode sol.
24	1	2	1
48	2	3	2

When Sarcoma 37 tissue had been in contact for 4 hours with a concentration of 80 μg., or even 45 μg., of A-2Y per milliliter in saline, and was then transferred to the basic culture medium (75 cultures per solution), it showed a pronounced increase in diameter as compared with tissue previously immersed in saline for the same length of time (Table 2). This increase, regularly observed,

TABLE 2

RELATIVE INCREASE IN DIAMETER OF EXPLANTS IN CONTROL NUTRIENT OF S-37 TISSUES PREVIOUSLY IMMERSED FOR 4 HOURS IN SOLUTIONS CONTAINING A-2Y AND A-2 AT DIFFERENT CONCENTRATIONS

(75 cultures in each solution)

	A-2Y, μg/ml			A-2, μg/ml		Control Tyrode
Hours	45	80	450	80	800	sol.
24	2	2	1	2	1	1
48	3	3	2	3	2	2

was of the same order as for the chicken fibroblasts. No increase was noted after contact with 450 μg. of A-2Y. This was likewise true when the tissue was immersed in concentrations of 80 μg. and 800 μg. of A-2, 80 μg. resulting in increased diameter, 800 μg. being without effect (Table 2).

When A-2Y was introduced into the medium in concentrations of 3.3, 20, or 33 μg/ml, it failed to modify the increase of Sarcoma 37. Slight increase resulted when the concentration was held at 330 μg/ml (Table 3).

TABLE 3

RELATIVE INCREASE IN DIAMETER OF EXPLANTS OF S-37 TISSUES IN MEDIA CONTAINING A-2Y

(50–75 cultures in each solution)

	μg/ml			
Hours	3.3	20	33	330
24	1	1	1	1½
48	2	2	2	2½

DISCUSSION

In view of the fact that certain bacterial polysaccharides, when injected into mice, induce symptoms of shock followed, in tumor-bearing animals, by hemorrhage and necrosis of the tumor, it might be expected that they would have a damaging effect on isolated tissue. However, in agreement with McConnell's observation (4), we could see no evidence of damage, as indicated by fatty degeneration, bleb formation, or excessive cell death, when such polysaccharides were applied to tissue cultures of chick heart fibroblasts or of mouse Sarcoma 37 cells. On the contrary, the striking fact brought out by these experiments was the ability of appropriate concentrations of the polysaccharide preparations to enhance the areal increase of chick heart fibroblasts and Sarcoma 37 cells in culture.

There is sufficient variation in the tumor-necrotizing properties known to exist between different polysaccharide preparations to make it dangerous to assume without direct evidence that two preparations known to possess like properties with regard to their effects on tissue cultures will also possess like necrotizing effects. However, the results of these experiments nevertheless make it appear doubtful that the toxic effects such as hemorrhage and tumor necrosis, which follow the administration of polysaccharide to mice or human patients, can be attributed to any direct toxic action of the polysaccharide on the tumor. Algire (1) considers, rather, that the damage done to tumors is secondary to vascular damage and the resulting anoxia of shock. It is possible that there may also be intermediate products of metabolism responsible for the toxic effects observed.

It is not clear how the observed enhancement of areal increase following treatment with bacterial preparations is brought about. It is possible that there is an enzymatic breakdown of polysaccharide to simple sugars which supply energy to the tissue. Such complexes sometimes provide detoxifying mechanisms for the removal of products of tissue metabolism.

SUMMARY

The effect of bacterial polysaccharide fractions isolated from *Serratia marcescens* and *Aerobacter aerogenes* has been studied on tissue cultures of normal chicken fibroblasts and of cells of Sarcoma 37 from mice. These fractions are not directly toxic for the tissues but may rather enhance the areal increase of both types of tissue.

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Use of Serum Proteins in Measuring Activity of Prostatic Cancer*

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We have observed that the serum proteins of patients with advanced prostatic cancer rapidly return to or toward normal values following deactivation of the neoplasm by anti-androgenic procedures. While there exists a variety of methods, more or less satifactory, for observing these changes, perhaps none is so simple or so useful as the determination of the lowest percentage of blood serum to gel after heating under standard conditions. The use of this assay method is the subject of the present paper.

One of the central problems in the chemotherapy of cancer is to obtain methods of measuring neoplastic activity and the effects of medicines thereon, with precision and sensitivity, preferably with simplicity. Advanced prostatic carcinoma of man is peculiarly adaptable for quantitative studies of activity and, conversely, partial or complete inactivation of the tumor by therapeutic agents. In the past this neoplastic activity could be measured by two modalities which reflected local events in and near the carcinoma. When this neoplasm has metastasized, the level of acid phosphatase in serum is often increased (3). A common site of metastasis of this tumor is bone marrow, a tissue wherein the neoplasm thrives and usually stimulates the growth of osteoblasts, which process in turn results in an elevation of alkaline phosphatase (9) in the serum. When one investigates (7) these enzymatic levels in the blood concurrently over a period of days, he measures the relative production of acid phosphatase by the metastases (an activity of the cancer) and the response of adjacent nonmalignant bone tissue to the presence of that tumor (local reactivity of normal host cells). Unlike these measurements of essentially local changes, albeit occurring in many places, a determination of the least percentage of thermocoagulable protein reflects the over-all state of certain plasma proteins, especially albumin, and hence is an indicator of the efficiency of general physiologic mechanisms and balances.

It has been shown (6) that the transformation of serum under the influence of heat to form a cohesive jelly depends largely, if not exclusively, on its content of serum albumin. Changes in serum albumin frequently occur in patients with cancer, but unfortunately no method exists whereby the quantity of albumin in serum may be determined with precision. The simple determination of the quantity of albumin present does not completely characterize this protein, because albumin possesses the property of binding anions to itself (2), a peculiarity only shared by certain rare γ-globulins among the serum proteins (8). Both the quantity of protein and certain associated anions such as desoxyribonucleate (1) affect the thermocoagulability of albumins.

The determination of the lowest thermocoagulable percentage of serum is a simple and highly reproducible physical method of investigation of serum proteins. Its determination yields a primary value. The implications of the results from the standpoint of physical chemistry are complex. In our experience with clinical patients a decrease in the least coagulable percentage of serum always means an improved physiologic state, while a higher percentage always implies deterioration. Its determination is one objective way of measuring general damage or freedom therefrom, but it is unspecific with respect to the nature of the damaging agency.

METHODS

Nineteen patients with untreated cancer of the prostate in this series were classified in two categories; (*a*) early cancer was defined as small nodular carcinomas confined to the anatomical limits of the prostatic gland; (*b*) advanced cancer included all cases with metastases or appreciable extension outside of the site of origin.

The following determinations were made on the blood serum of the patients: total protein by the

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micro-Kjeldahl technic of Ma and Zuazaga (11); albumin after precipitation of globulins with 23 per cent sodium sulfate by use of the Weichselbaum (13) modification of the Howe (5) method; acid and alkaline phosphatases by the method of King and Armstrong (10); anion binding of serum with the equilibrium dialysis of phenolsulfonephthalein (8); and least coagulable percentage of serum (6).

The upper limit of values derived from examination of the sera of more than 500 normal adults in this laboratory are for acid phosphatase, 4.5 King and Armstrong units per 100 milliliters of serum; for alkaline phosphatase, 11 units; least coagulable percentage of serum, 20 per cent.

In certain cases diethylstilbestrol was administered intramuscularly in solution in sesame oil; orchiectomy was done under local anesthesia.

OBSERVATIONS

Serum—least coagulable percentage and level of phosphatases.—The results are summarized in Table 1. There were twelve cases in this series (63 per cent) with elevated values for thermal coagulation percentage; of the seven men with normal coagulable percentage, one patient had elevated phosphatases. There were six cases in the series (32 per cent) with elevated phosphatase levels, one of whom had normal coagulation values. Thus, five patients had both elevated phosphatase and coagulation findings; four cases of advanced prostatic cancer had both normal phosphatase and coagulation values.

TABLE 1

ACID AND ALKALINE PHOSPHATASES AND LEAST COAGULABLE PERCENTAGE OF SERUM IN 19 UNTREATED CASES OF CANCER OF THE PROSTATE

		ACID AND ALKALINE PHOSPHATASES		LEAST COAGULABLE PERCENTAGE	
	No.	Within normal limits	Elevated	20 per cent or less	Greater than 20 per cent
Early cancer	3	3	0	2	1
Advanced cancer	16	10	6	5	11

The data show that elevated coagulation percentage values of serum are more common in cancer of the prostate than elevation of acid and alkaline phosphatases.

The effect of orchiectomy on the least coagulable percentage of serum.—Bilateral orchiectomy was performed 10 times in patients with elevated coagulation values. Without exception there was a decrease in coagulation percentage within 2–5 days; a typical example is shown in Chart 1. The changes in the coagulation percentage were usually spectacular and were evident at an earlier period than significant changes in total protein or albumin content.

The effect of diethylstilbestrol on least coagulable percentage and phosphatases of serum.—This estrogen was administered intramuscularly to five patients. In 4 cases the least coagulable percentage of serum decreased (Chart 2), although less rapidly than after orchiectomy, the first improvement being observed after 8–10 days of treatment. In one case (Chart 3), stilbestrol induced massive retention of water with anasarca; although the activity of the cancer was inhibited, as was shown by the serum phosphatases, the clinical condition

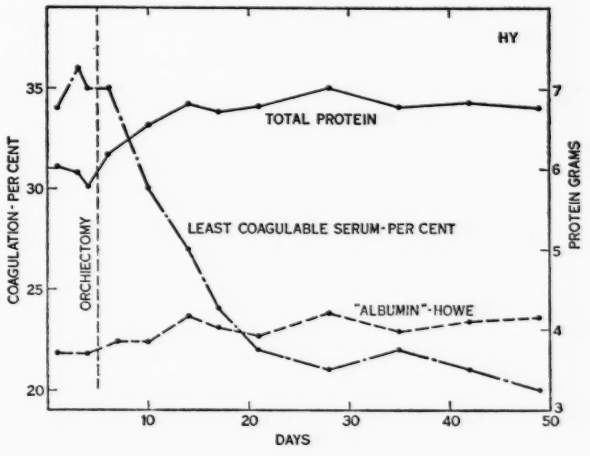

CHART 1.—Effect of orchiectomy on the least coagulable serum percentage, total protein and albumin as determined by the Howe method (5).

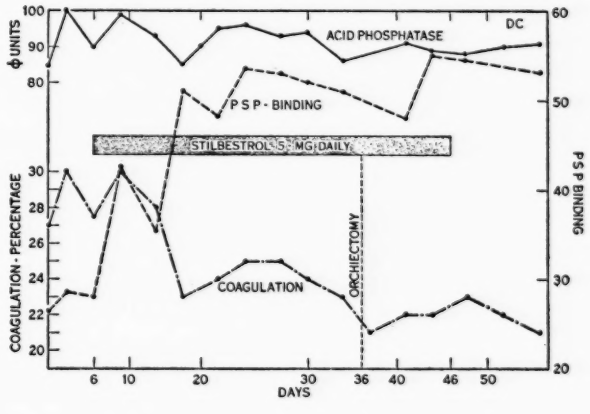

CHART 2.—Effect of diethylstilbestrol and orchiectomy on acid phosphatase content, the binding of phenolsulfonephthalein (8) and on least coagulable percentage of serum. The clinical condition of this patient with prostatic cancer was greatly improved by the anti-androgenic therapy, and the amelioration was reflected in the increase of anion binding and the decrease of the coagulation percentage; no striking change however occurred in the acid phosphatase level.

of the patient became worse, and this was reflected in the coagulable percentage. In all patients in the series changes in coagulable percentage coincided with the clinical evaluation of the case.

In an earlier paper (7) it was shown that prostatic cancer could often be deactivated by injec-

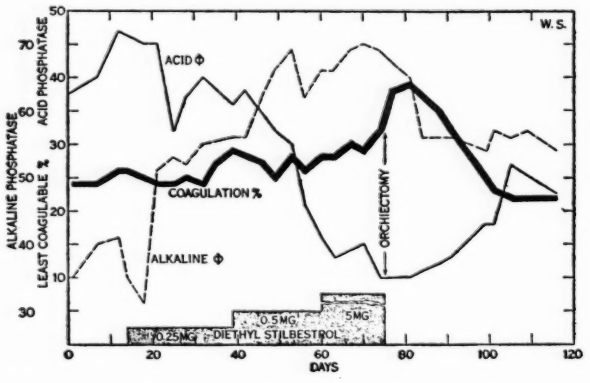

CHART 3.—Changes in acid and alkaline phosphatases and least coagulable percentage of the serum of a man with advanced prostatic cancer treated with diethylstilbestrol, intramuscularly, and later by orchiectomy. The dosage of diethylstilbestrol is given in milligrams per day. While the drug induced changes in the phosphatases characteristic of deactivation of the neoplasm, it caused retention of water and a general debility of the patient, which was reflected in the findings in thermal coagulation.

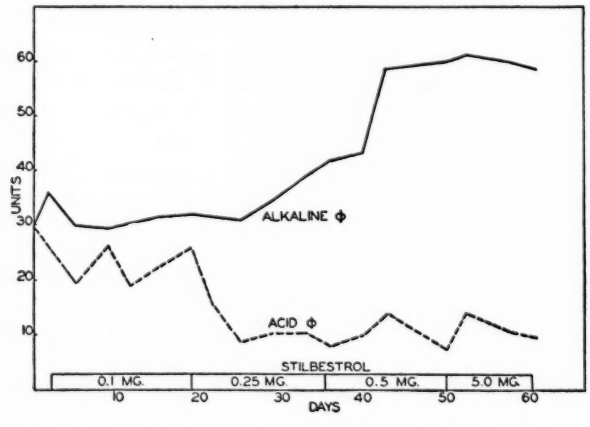

CHART 4.—Effect of increasing doses of diethylstilbestrol on acid and alkaline phosphatases in cancer of the prostate. The results are expressed in King and Armstrong (10) units per 100 milliliters of serum.

tions of stilbestrol, 1 mg. daily; the minimal effective dose of this estrogen was not sought for at that time, nor has it been reported subsequently. In the present series stilbestrol was administered proceeding from ineffective to effective amounts, each dose level being maintained for 14 days. The quantitative criteria of effectiveness were acid and alkaline phosphatase changes and a decrease in thermal coagulation percentage. In patients with osseous metastases the phosphatase changes indicative of deactivation are a decrease of acid phosphatase with a simultaneous rise of alkaline phosphatase (Chart 4). In each of three patients who were studied in this way, 0.1 mg. of diethylstilbestrol administered daily was ineffective, while 0.25 mg. of this agent induced a remission. The minimal effective dose of diethylstilbestrol in human prostatic carcinoma, therefore, is in the vicinity of 0.25 mg. when injected intramuscularly each day. Increasing the dose level to 5 mg. daily did not increase the therapeutic efficiency of this estrogen.

DISCUSSION

In a series of patients with gastric cancer subjected to a removal of the tumor, it was found (4, 12) that the abnormal α-globulin and fibrinogen values returned to normal within a few weeks after the surgical operation, while an increase of serum albumin was extremely slow even in patients maintained in positive nitrogen balance. By contrast in the present series of patients with advanced prostatic cancer, the least coagulable percentage of serum improved within a few days after anti-androgenic control.

CONCLUSIONS

Changes in nonenzymatic plasma proteins are more common in advanced prostatic cancer than are elevations of acid or alkaline phosphatases. These changes were observed simply and accurately by determining the least thermocoagulable percentage of serum. An increased percentage always indicated deterioration of physiologic mechanisms, and a decreased percentage reflected improvement. Usually following anti-androgenic control of the cancer, the least coagulable percentage of serum returns rapidly toward normal values.

Orchiectomy induces changes in nonenzymatic proteins of the serum more rapidly in cancer of the prostate than the administration of estrogen. The minimal effective dosage of diethylstilbestrol in human prostatic cancer is close to 0.25 mg. daily when this medicine is administered intramuscularly; increasing the dosage to 5 mg. daily did not enhance the effect of this estrogenic substance.

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A Comparison of the Retardation of Sarcoma 180 by SK 1424, 3-Bis (β-chloroethyl) aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine with That by HN2, Methylbis (β-chloroethyl) amine*†

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Many profound disturbances in widely diversified biological systems have resulted from exposure of the biological material to nitrogen mustards (7, 17). Prominent among the actions of nitrogen mustards is their ability to produce leukopenia; this was first observed during studies of the pathological changes induced in experimental animals exposed to chemical warfare agents (5).[1] Subsequent studies on the action of HN2 against human lymphosarcomas have provided evidence that the compound is a useful agent for the palliative treatment of certain forms of neoplastic disease (1, 9, 12, 15, 18). Related compounds have been studied in experimental animals at a number of laboratories in attempts to improve upon the limited usefulness of HN2 (1, 3, 4, 11, 19–21).

The results of our early experiments with HN2 and Sarcoma 180 suggested that mouse leukemia would be a better indicator of anti-tumor activity among the nitrogen mustards. After the relative effectiveness of a number of mustards against mouse leukemia (3, 4) had been studied, the experiments were extended to include Sarcoma 180. This communication reports a nitrogen mustard which has shown greater ability than HN2 in the retardation of Sarcoma 180. The results with a large number of nitrogen mustards will be presented in a later survey.

MATERIALS AND METHODS

The compounds prepared for this study were characterized by elementary analysis.[2] The mustards and most of the other compounds were tested as solutions prepared just prior to use. The amounts injected initially were based on the maximum doses tolerated on repeated injections, as determined routinely prior to use in tumor-bearing animals.[3] Mice (CFW Swiss females, 18–22 gm.) received subcutaneous implants by the usual trocar method. Twenty-four hours later, intraperitoneal injections of the compounds were started and continued once or twice daily for 7 days. At the end of the course of injections the diameters of the tumors were measured as described previously (16). The degrees of inhibition were graded as follows:

+ = The diameter was $\frac{1}{4}$, or less, of that of the control tumors; actually, the volume of such a tumor would be $\frac{1}{64}$, or less, of the control if tumors may be considered spherical.

± = The diameter was $\frac{1}{4}$ to $\frac{3}{4}$ of the diameter of the control tumors.

− = The largest diameter was $\frac{3}{4}$ or more of the diameter of the control tumors.

In some experiments the mice were observed for several additional weeks for indications of delayed toxicity to the host and permanent damage to the tumors.

The histo-pathological effect of SK 1424 on Sarcoma 180 and the general cytotoxic actions, including effects seen in peripheral blood, were investigated in a special cytological experiment. Tumor-bearing mice were sacrificed for the tissue studies after 3, 5, and 7 days of treatment with SK 1424 (5 mg/kg/day administered fresh daily).

DISCUSSION OF RESULTS

The data from the tumor retardation studies of a number of substituted pyridines are presented in Table 1. From this it can be seen that only SK 1424 and SK 2032 possess significant ability to retard Sarcoma 180. These compounds were pre-

* We wish to acknowledge support of this study by funds from the American Cancer Society.

† With the technical assistance of Margaret L. Keeve and Angela T. Boryczka.

[1] C. Lushbaugh, unpublished data.

[2] We are indebted to Drs. Max Tishler and Evelyn Wilson, Merck & Co., Inc., for synthesis and provision of generous supplies of all the compounds presented in this report.

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[3] F. S. Philips, unpublished data.

TABLE 1

ABILITY OF SELECTED PYRIDINES TO INHIBIT THE DEVELOPMENT OF SARCOMA 180 IN MICE

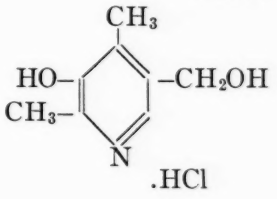
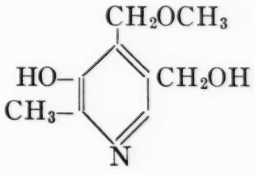
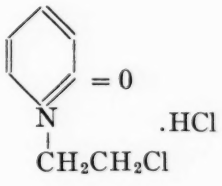
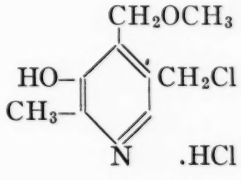
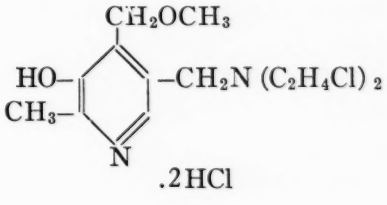
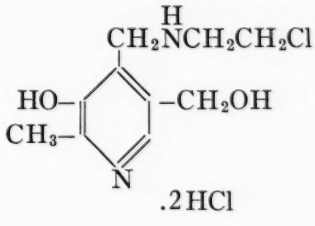
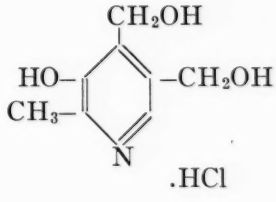
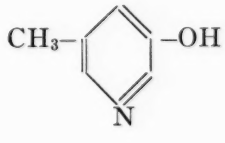
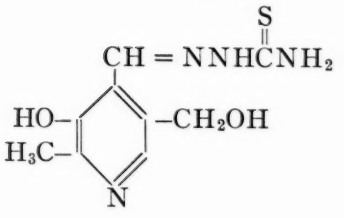
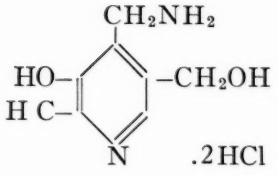
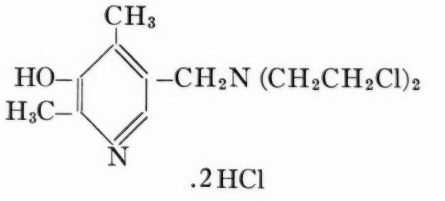
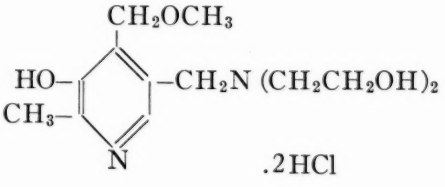
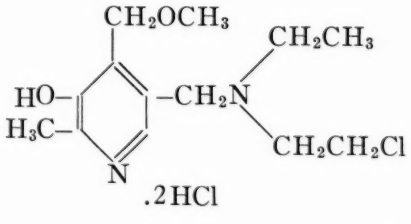
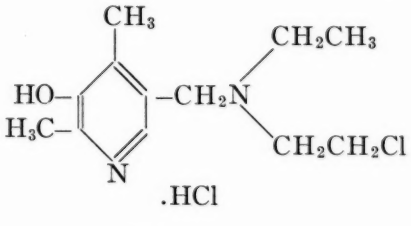
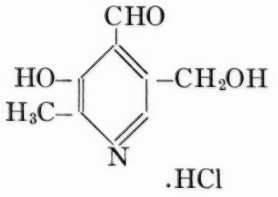
SK no.	Name	Structure	Dose level mg/K/day	No. deaths/ No. animals	Tumor inhibition
591	4,6-Dimethyl-5-hydroxy-3-hydroxymethylpyridine hydrochloride (Desoxypyridoxine)		128–200 225	34/95 5/15	− to ± − to ±
778	3-Hydroxymethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine (Methoxypyridoxine)		32–40 50	3/35 6/15	− −
1422	N-β-Chloroethylpyridone-2 hydrochloride		125–150 200	0/15 2/20	− −
1423	3-Chloromethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine hydrochloride		250–350 400	0/25 0/5	− −
1424	3-Bis(β-chloroethyl)aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine dihydrochloride (Methoxypyridoxyl nitrogen mustard)		2.5 5.0 7.5 10 15–16	0/20 2/25 4/45 2/60 2/50	− to ± ± ± to + + +
1447	3-Hydroxymethyl-4-chloroethylaminomethyl-5-hydroxy-6-methylpyridine dihydrochloride (Pyridoxyl nitrogen mustard)		125–150 175	1/15 4/10	− −
1547	3,4-Bis(hydroxymethyl)-5-hydroxy-6-methylpyridine hydrochloride (Pyridoxine)		250–1,500 2,000	1/30 1/5	− −
1586	5-Methyl-3-pyridol		250	2/10	−

TABLE 1—*Continued*

SK no.	Name	Structure	Dose level mg/K/day	No. deaths/ No. animals	Tumor inhibition
1996	Pyridoxyl thiosemicarbazone		250	6/15	–
2031	3-Hydroxymethyl-4-aminomethyl-5-hydroxy-6-methylpyridine dihydrochloride (Pyridoxamine dihydrochloride)		500	1/5	–
2032	3-Bis(β-chloroethyl)aminomethyl-4,6-dimethyl-5-hydroxypyridine dihydrochloride (Desoxypyridoxyl nitrogen mustard)		1 2–5 6 7 8	0/20 8/90 12/55 1/10 3/5	– ± ± to – ± ?
2111	3-Bis(β-hydroxyethyl)aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine dihydrochloride		500–1,000 1,500	2/30 0/5	– –
2182	3-(N′-Ethyl-N′-β-chloroethyl)aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine dihydrochloride		16–24 30	2/25 3/10	– –
2219	3-(N′-Ethyl-N′-β-chloroethyl)aminomethyl-4,6-dimethyl-5-hydroxypyridine hydrochloride		24–30 32	6/30 6/15	– to ± – to ±
2259	3-Hydroxymethyl-4-formyl-5-hydroxy-6-methylpyridine hydrochloride (Pyridoxal hydrochloride)		250	0/5	–

pared with the hope that the bis(β-chloroethyl)-amines substituted with natural groups, such as amino acids or vitamins, might possess interesting properties. SK 1424 and SK 2032 represent nitrogen mustards in which the third substituent is a pyridoxine analog. Desoxypyridoxine, SK 591, shows a slight activity in spite of maintenance of the test animals on a normal diet. In an earlier report (22), a pyridoxine-deficient diet was necessary to show an anti-tumor activity of desoxypyridoxine. It is worth noting that methoxypyridoxine, SK 778, does not show this activity, even though tested at relatively toxic levels. An explanation for the difference may be found in studies which indicate that methoxypyridoxine in the mouse is not an antipyridoxine and may be utilized to provide pyridoxine (6).

chromatin. In other instances they were karyorrhectic or pyknotic. The picture was similar to that observed after treatment with other nitrogen mustards (14).

Table 2 presents a comparison of the inhibiting capacities of SK 1424 and HN2. It appears from these data that for doses comparable in early or delayed toxicity SK 1424 has shown greater tumor retardation. Whether the increase in anti-tumor activity is due to the specific effect of the methoxypyridoxine group or to the general influence of an aromatic substituent is undecided. Other studies have indicated some improvement in the anti-tumor abilities of the nitrogen mustards through aromatic substitution (11).[4]

As HN2 has taken its place in the treatment of certain types of neoplastic disease, it would seem

TABLE 2

INHIBITION OF SARCOMA 180 BY SK 1424 AND BY HN2

Compound	Conc. mg/K/day	No. groups of 5 mice tested	Graded results* No. of Groups Showing +	±	−	Toxicity data† deaths in (7 days)	(4 weeks)
HN2	0.8	12	0	8	4	3/60	16/60
	1.0	5	0	2	3	4/25	6/25
	1.2	5	0	5	0	5/25	17/25
	1.4	5	2	2	1	3/25	17/25
SK 1424	2.5	4	0	2	2	0/20	0/20
Fresh solutions	5.0	5	0	5	0	2/25	8/25
	7.5	9	4	5	0	4/45	9/45
	10.0	12	9	3	0	2/60	27/60
	15.0	10	10	0	0	3/50	24/50
SK 1424	10	3	0	0	3	0/15	
Old solutions	15	5	0	5	0	0/25	8/25
	20	16	15	1	0	6/80	41/80

* No. of groups of mice showing the degree of tumor inhibition

† Numerator shows number of deaths. Denominator shows the total number of test animals.

A number of substituted pyridines were tested in a search for additional active compounds as well as for further information relative to the activity of SK 1424 and SK 2032. It was observed that SK 2111, the hydrolysis product of SK 1424, is inactive. SK 2182 and SK 2219, the monochloroethyl analogs of SK 1424 and SK 2032, respectively, are inactive. This finding is consistent with other studies which have shown that anti-tumor properties are lacking in most nitrogen mustards with only one β-chloroethyl group.

The mice treated with SK 1424 showed a leukopenia and damage to the gastro-intestinal tract, changes which correspond to the findings described earlier for the nitrogen mustards (10). Sarcoma 180 in the treated animals exhibited a marked increase in necrobiotic processes and a decrease in mitosis in comparison to untreated control tumors. SK 1424 caused the appearance of numerous giant cells, the cytoplasm of which showed peripheral vacuolar degeneration. The nuclei were in some instances enlarged and appeared to contain less

worth while to call attention to any related compound that appears superior in action against experimental animal tumors. On this basis SK 1424 has been given preliminary clinical trials in fifteen to twenty patients (13). In intravenous doses of 1 mg/kg/day for 5 days, it has caused leukopenia, dizziness, and a low incidence of nausea and vomiting. As nearly as could be judged by only ten patients, its toxicity and therapeutic effectiveness were proportional, and its range of activity against different types of tumors was parallel to that of HN2. SK 1424, in oral doses of 2–4 mg/kg/day, caused severe nausea, vomiting, and loss of taste or a persistent bad taste. There was little evidence of leukopenia or therapeutic effect from the oral doses. Loss of taste and neurological symptoms have been observed before from certain β-chloroethyl amines (1, 2, 8). Therefore, although it has not been adequately explored, it is doubtful that large doses of pyridoxine would be useful in combatting the adverse effects of SK 1424. The desira-

[4] C. Chester Stock, unpublished data.

bility of further clinical trials with SK 1424 would appear to be diminished as a result of the developments in clinical trials with SK 1133, 2,4,6-tris-(ethylene imino)-*s*-triazine (13).

SUMMARY

A nitrogen mustard, SK 1424, has been found which appears to be more efficient in retarding Sarcoma 180 than HN2, the nitrogen mustard used as our standard.

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The Effect of 8-Azaguanine on Physiologic Growth Measured by the Rate of Eruption of the Incisor of the Mouse*

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Following his demonstration that guanine is a metabolic requirement in Tetrahymena, Kidder (2) showed that guanine deficiency, brought about through competitive inhibition by the structural analogue 8-azaguanine, resulted in inhibition of the growth of Tetrahymena colonies. It had previously been shown by Brown *et al.* (1) that guanine is not a nutritive requirement for rats. Guanine fed to rats is not used as raw material for the synthesis of nucleic acids but is broken down and excreted (1). Kidder reasoned that, if tumor metabolism resembled that of ciliates rather than that of normal mammalian tissues, then guanine might be a dietary requirement for tumor growth and 8-azaguanine might inhibit the growth of tumors by competitive inhibition, while not affecting normal tissues.

Tests in mice (2) showed that the subcutaneous or intraperitoneal administration of 8-azaguanine had a very pronounced action in retarding the growth of adenocarcinoma E 0771 and of several other tumors, while not seeming to affect the health of control animals.

It seemed interesting, therefore, to test Kidder's hypothesis that the growth-inhibiting action of 8-azaguanine is limited to tumor tissue. For this purpose, a more sensitive method was required than the commonly used procedures of following weight curves or of determining "therapeutic ratios" (3). It was hoped that a study of the rate of incisor eruption might prove to be a better tool. As is known, the incisors in rodents are continuously erupting teeth. While the peripheral end shears off, the same over-all length is maintained through continuous growth at the open basal end and through eruption of the tooth. The rate of eruption, therefore, is a direct measure of rate of growth of the tooth. It was felt that the rate at which growth of the tooth proceeds is likely to be indicative of the physiological state and the general conditions for growth in the animal. An appraisal of the method will be presented in some detail below.

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MATERIALS AND METHODS

Seventy-eight mice of strain C57 black, obtained from the Roscoe B. Jackson Laboratories in Bar Harbor, Maine, were used. The animals were kept on a diet of Ralston Purina Fox Chow checkers and water *ad libitum*. The rate of incisor eruption was observed in the following groups of animals:

a) Control groups V and VI, consisting of ten and eleven animals, were left untreated. Control group VII, ten animals, was given Locke's solution in two daily injections of 0.5 cc. each. All control animals were 7–8 weeks old at the beginning of observation.

b) Experimental group III consisted of eighteen animals 7–8 weeks of age at the beginning of the experiment, and Group O of nine animals, aged 10–11 weeks. Experimental group II contained nine animals 7–8 weeks of age, into which adenocarcinoma E 0771 had been transplanted. 8-Azaguanine, obtained by courtesy of the Lederle Laboratories Division of the American Cynamide Company, was dissolved and administered to the experimental animals as suggested by Kidder, i.e., twice daily, either subcutaneously or intraperitoneally, in quantities of 0.5 mg. in 0.5 cc. of solution per injection.

The rate of eruption was measured in one or both lower incisors of control and experimental animals. A shallow scratch was applied to the tooth with a carborundum disc rotating in a dental engine handpiece. The distance between the mark and the gingival margin was measured once a week with calipers calibrated in tenths of millimeters and a fresh mark applied when necessary.

The animals did not have to be anesthetized for this procedure.

RESULTS

Appraisal of Method

Error of measurement.—To evaluate the accuracy with which the weekly rate of eruption could be measured in mice, double determinations, on right and left incisors, respectively, were carried out during a 4-week period. The averages for right incisor eruption and left incisor eruption were found to differ by 0.08, 0.02, 0.10, and 0.05 mm. in the 4 successive weeks. In every instance this difference was smaller than the standard error of each average, which was found to range from 0.07 to 0.13 mm.

The error of measurement, therefore, is small enough to permit reliable averages in fairly small groups of animals. As regards the reliability of individual measurements, the standard deviation for the series of discrepancies between double measurements is 0.280 mm.; and for an individual measurement, 0.20 mm. The odds, therefore, are as 22 : 1 against an individual rate being more than 0.4 mm. distant from the true value.

Variation between individuals of the same age.—In the three control groups summarized in Table 1, there was found to be a rather close agreement with respect to upper and lower extremes of rate, weekly average for each group, and variability (standard deviation). In the first week, e.g., the extremes in Group VI (including right and left measurements) were 1.2 mm. and 2.4 mm.; in Group V, 1.1 and 2.3; in Group VII, 1.4 and 2.1 mm. The weekly averages for the three groups were 1.71, 1.72, and 1.79, respectively, and the standard deviations 0.39, 0.41, and 0.36. The standard error of the mean for the same three groups was found to be 0.12, 0.13, and 0.12, respectively. Thus, fairly small to moderate effects on the rate of eruption can be tested for, without necessitating very large groups of animals.

TABLE 1

RATE OF ERUPTION OF INCISORS

Group	8-Aza-guanine	Week	No. animals	Av. eruption (mm.)	Std. error	Std. deviation	Mean weight (gm.)	Deaths in week
VII	0	1st	10	1.79	0.12	0.36	18.7	None
V	0	"	10	1.72	0.13	0.41	16.9	None
VI (Right)	0	"	11	1.67	0.13	0.42	18.5	None
VI (Left)	0	"	11	1.75	0.11	0.36	18.5	None
III	+	"	18	1.04	0.07	0.28	19.3	None
O	+	"	9	0.96	0.13	0.39		None
II	+	"	9	1.21	0.12	0.37		None
VII	0	2d	10	1.66	0.12	0.38	17.6	None
VI (Right)	0	"	11	1.82	0.15	0.49	18.9	None
VI (Left)	0	"	11	1.80	0.13	0.42	18.9	None
III	+	"	18	1.02	0.09	0.39	17.0	None
O	+	"	9	1.32	0.17	0.50		None
II	+	"	9	1.03	0.14	0.36		None
VII	0	3d	10	1.58	0.10	0.31	18.2	None
VI (Right)	0	"	11	1.61	0.14	0.46	18.9	None
VI (Left)	0	"	11	1.51	0.12	0.41	18.9	None
III	+	"	16	1.36	0.07	0.28	18.4	1
O	+	"	8	1.85	0.15	0.42		None
II	+	"	8	1.44	0.18	0.50		
VII	0	4th	10	1.60	0.17	0.53	19.0	None
VI (Right)	0	"	11	1.59	0.09	0.30	20.3	None
VI (Left)	0	"	11	1.64	0.07	0.24	20.3	None
III	+	"	14	1.10	0.10	0.38	17.6	2
O	+	"	8	1.24	0.14	0.38		None
II	+	"	7	0.81	0.14	0.38		

Variation in time, in the same animal.—Two groups of control animals were observed for a period of 4 weeks (one untreated, the other receiving twice daily an injection of 0.5 cc. of Locke's solution). There were, in a total of 88 measurements of the rate of incisor eruption, seven instances of an upward shift of 0.7 mm. or more, six instances of a downward shift of 0.7 mm. or more, four instances of an upward shift between 0.4 and 0.7 mm., and twelve instances of a downward shift between 0.4 and 0.7 mm. Above, the standard deviation of an individual measurement was computed to be 0.2 mm. Thus, in the 29 instances of shifts of 0.4–0.7 mm., we are very probably dealing with biological variations rather than shifts caused by error of measurement. Gains and losses in rate balanced, to a certain extent. However, the preceding data as well as the weekly averages (Ta-

ble 1 and Chart 1) show that there was a slight downward trend in the rate of eruption of our control animals (probably caused by somewhat unfavorable conditions occurring in our animal hospital at the time of the experiment), which in the case of Group VI was pronounced enough to be statistically significant.

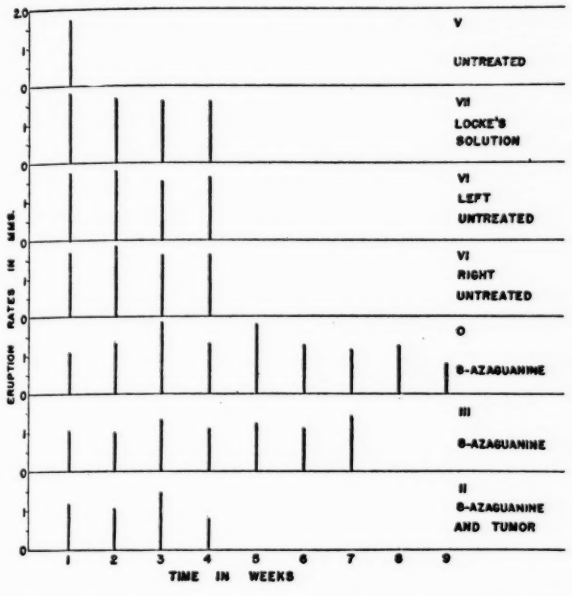

CHART 1.—Average weekly rates of eruption of the lower incisors in control and experimental animals.

In using the rate of incisor eruption as a test object, it is therefore preferable to carry out control measurements in a group of untreated animals kept under identical conditions rather than to establish "before and after" values in the test animals.

THE EFFECT OF 8-AZAGUANINE ON THE RATE OF ERUPTION

The effect of 8-azaguanine treatment was studied in three groups of animals: a small trial group, a larger group of somewhat younger animals, and a group of animals into which tumor transplants had been made (Groups O, III, and II of Table 1). This latter group is included for discussion in this paper because the values found for incisor eruption during the first 4 weeks of the administration of 8-azaguanine were very similar to those found in the other two groups, despite the presence of the tumor.

Effect during fourth to eleventh day of treatment.—A strongly depressing effect of 8-azaguanine during the first week of its administration was unmistakable. The average rate of eruption found in the three groups was 1.04, 0.96, and 1.21 mm., respectively, as compared to 1.71, 1.72, and 1.79 for the three control groups or 1.74 for all control animals taken together. The difference between the average for all control animals and the average for Group III was 0.70 ± 0.089, or 7.8 times its standard error; for Group O, 0.78 ± 0.144, or 5.4 times its standard error; for Group II, 0.53 ± 0.136, or 3.9 times its standard error. Chart 2 shows the percentage distribution of the animals over the range of rates of eruption. The distribution curves for the three groups of animals receiving 8-azaguanine are shifted toward the lower rates, showing that the averages were depressed because the rate of eruption was depressed in most, if not all, animals. In fact, of the 36 animals receiving 8-azaguanine, there was only one (with a rate of 1.7 mm.) in the region of the average of the controls (1.74). There was one animal with a rate of 1.6 mm. and five with rates of 1.5 mm. Of the seven animals just mentioned, five showed very depressed rates in the second week of observation, and one showed by its higher later readings that 1.5 mm. was a depressed rate for this animal. Only one animal with an eruption rate of 1.5 gave no evidence of being affected by 8-azaguanine administration.

The length of time for which 8-azaguanine had to be administered before its full inhibitory effect could be seen varied in different animals. Thus, not only in the five animals just mentioned but in eight additional ones with rates already low in the first week, there was a further slowing of eruption

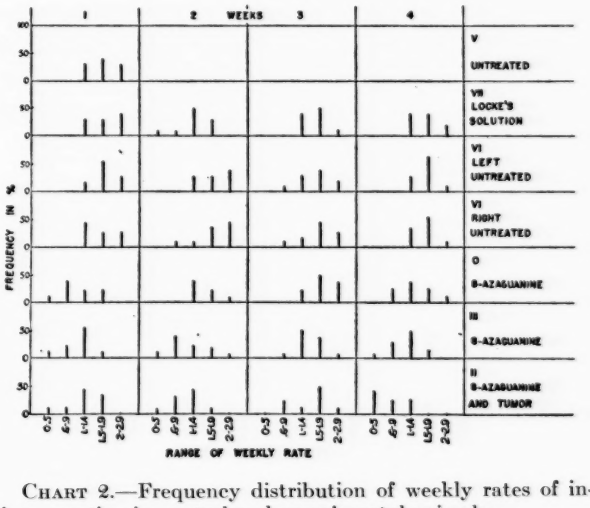

CHART 2.—Frequency distribution of weekly rates of incisor eruption in control and experimental animals.

in the second week, whereas in eleven animals maximal inhibition seemed reached during the first week of observation, and faster eruption occurred in the week thereafter. The averages shown in the table conceal these shifts, since gains and losses very nearly balanced in each of the three groups.

Later effects: recovery from 8-azaguanine.—While it can thus be stated that 8-azaguanine depressed the rate of tooth eruption in nearly all animals observed, a certain degree of "escape" from its effects while administration is continued seemed to be an equally universal occurrence. This recovery phenomenon was somewhat variable in time and degree and was, in a majority of the animals, transient in character. Among the animals surviving for 3 weeks or more, there were only two in which the rate of eruption in later weeks did not rise beyond that measured in the first and/or second week. One of these was the animal unaffected by 8-azaguanine mentioned in the preceding section, the other one was the only animal with a consistently low rate which we have observed.

a) Timing: Most commonly, the "escape" occurred in the third week of administration. (For this reason, the group averages for this week show an increase in all three groups; see Table 1.) Of the 32 animals observed for this length of time, 21 showed higher readings in the third week than in the second week and in the fourth week, 7 had a peak rate in the second week already, 2 in the fourth week, and 2 in the fifth week.

b) Degree: As regards degree of recovery, values well within the control range are reached by all animals of Group O (a group of somewhat older animals), and values very close to it by all but two of the surviving animals of Groups III and II.

c) Duration: Recovery from the effects of 8-azaguanine administration was transient, lasting usually for 1, in some cases for 2, weeks. In all but one case it was followed by renewed depression, usually at least as marked as the initial one. However, most commonly this depression was again followed by recovery, very often to rates higher than those of the initial recovery phase. Although the number of animals observed for this length of time is small and the timing of the ups and downs varied in the different animals, the peculiar cyclical reaction to the continuous administration of 8-azaguanine was pronounced enough to be reflected in the weekly averages given in Chart 1.

This merely qualitative description of the effects of prolonged treatment is motivated by the small number of animals and the lack of control readings covering the same period. It was not possible to include Group II in this analysis, since animals with tumor E 0771, whether or not they are treated with 8-azaguanine, rarely survive for this length of time. However, even in the animals without tumors, particularly in the younger group (III), there was a rather high rate of mortality: one animal died in the third week, two in the fourth, five in the fifth, five in the sixth, one in the seventh week; the remainder survived. Among the older animals, only one died after 7 weeks of 8-azaguanine administration. There were no fatalities in the two control groups in the 4 weeks during which they were measured, nor in the following 8 weeks of observation.

DISCUSSION

Influence of weight changes on rate averages.—As the figures in Table 1 show, a pronounced tendency to lose weight was one of the effects of 8-azaguanine administration. Since this loss of weight may represent a diminished food intake, the decreased rates of eruption in the 8-azaguanine-treated animals might be ascribed to this factor rather than to a direct action of 8-azaguanine on the growth process in the tooth. The relationship between the rate of eruption and the weight of the animal was therefore analyzed. There was no correlation between rate and weight, nor was there (Chart 3) any relation between change in weight and change in rate. Rates lower than those of the preceding week were as frequently associated with a gain as with a loss in weight, and the same held for increased rates of eruption.

In the treated animals, there was also a random association of rate and weight. As regards weight *change*, small to moderate losses or gains showed random association with the large shifts in rate found in these animals (Chart 4). Thus, it was reported above that the rates of eruption observed during the second week of 8-azaguanine administration were partly higher, partly lower, and partly unchanged, as compared to those of the first week. Yet, during the same period, all except one stationary animal showed losses of weight ranging from 1 to 4 gm.

Only for the extreme weight changes observed was there a tendency for gains to be associated with gains and losses with losses in rate. Since even in this range of weight shifts this held only for the majority, and not for all the cases, loss of weight can at most be a factor which further depresses the rate of tooth eruption in the treated animals.

Influence of moribund animals on rate averages.—As was remarked before, there was a high mortality rate in the 8-azaguanine-treated animals. Many of the animals appeared to be sick many days before death (or recovery) occurred. The last rates of tooth eruption measured in these animals, however, showed as many instances of increase as of decrease over the preceding rate (10 decreases, 10 increases, and 2 stationary, in 22 animals dying during the period of 8-azaguanine administration). In only 2 of the 22 cases were ex-

ceptionally low rates found. However, both the last rate before death and the last but one rate before death tended to be lower than the respective weekly averages for the whole group. The

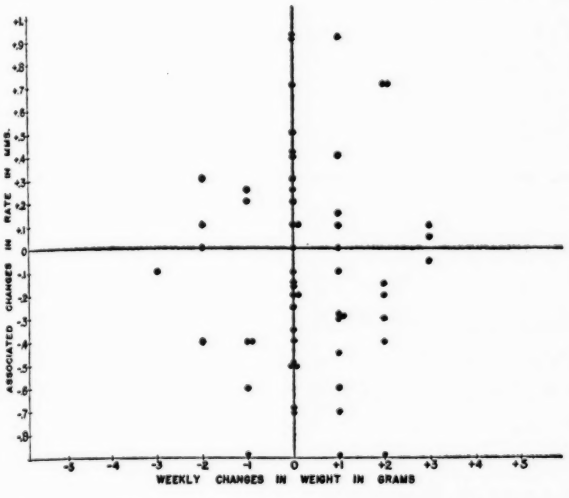

CHART 3.—Weekly changes in weight of control animals in their relation to changes in eruption over the same period.

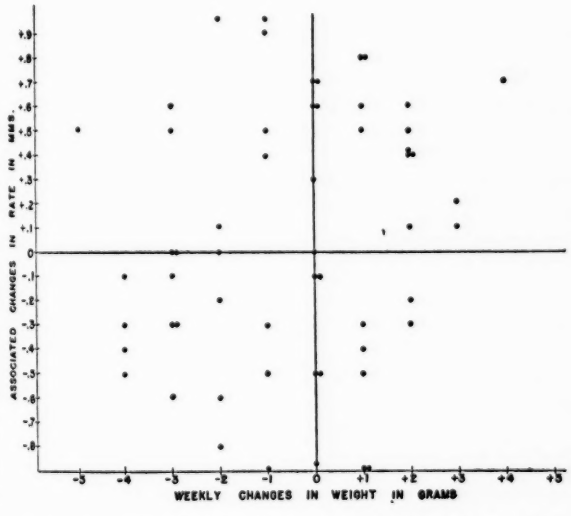

CHART 4.—Weekly changes in weight of experimental animals in their relation to changes in eruption over the same period.

inhibitive effect of 8-azaguanine on the rate of tooth eruption would, therefore, have appeared somewhat milder had those animals been excluded from the series which later succumbed to the treatment. But this, of course, would have meant excluding precisely those animals in which the systemic effect of 8-azaguanine was most pronounced.

The following comments and conclusions seem permissible:

The method of following the rate of incisor eruption in mice or rats[1] is useful in testing for the presence or absence and the severity of the effects of drugs on physiological growth. It seems of particular usefulness in the field of tumor chemotherapy where the goal is inhibition of a local growth process with—ideally—no effect on systemic growth processes.

The inhibitory effect of 8-azaguanine is not limited to tumor tissue. The fact that a drug also inhibits the growth of incisors and, therefore, presumably of other growing tissues, need not contra-indicate its use in tumor therapy. However, since the tumor-inhibiting action of a substance like 8-azaguanine does not outlast the period of administration, the concomitant inhibition of systemic growth might be of more serious concern.

SUMMARY

The rate of eruption of the lower incisors was studied in 78 mice of strain C57 black.

1. It was found that weekly rates of eruption can be measured with a degree of accuracy which permits reliable averages to be computed from fairly small groups of animals.

2. The rates of eruption found in different groups of untreated animals were found very similar in averages and range.

3. One ml. of Locke's solution per day, injected in two doses, had no significant effect on the rate of eruption.

4. 8-Azaguanine, in the dosage effective for pronounced inhibition of tumor growth in mice, markedly depressed the rate of incisor eruption in two groups of mice without tumors and in one group with transplanted adenocarcinoma E 0771.

5. Loss of weight was only partially responsible for this effect. It was concluded that the growth inhibiting effect of 8-azaguanine is not limited to tumor tissue.

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[1] Unpublished data of the Department of Dental Histology make it apparent that the rate of incisor eruption in rats is an equally reliable test object.

Proportion of Dietary Protein and the Formation of Spontaneous Hepatomas in the Mouse*†

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The incidence of spontaneously occurring benign hepatomas in C3H male mice is strikingly lower among animals fed a semi-purified ration containing 9 per cent casein than among those fed a diet containing 18 per cent casein as the principal source of protein (11). From the viewpoint of nutrition, the main differences between the diet containing 9 per cent and that with 18 per cent casein are (*a*) the total amount of protein and, more particularly, (*b*) the amount of specific essential amino acids, chiefly methionine and cystine; in a diet with 9 per cent casein these sulfur-containing amino acids are not present in amounts optimal for the growth of mice or the efficient utilization of the ingested ration. The following experiments were performed to determine whether either of these factors was responsible for the observed difference in hepatoma formation.

METHODS

The mice were inbred strain C3H males raised in our laboratories, and those used in a particular experiment were born within a 2-week period. They were distributed at random, approximately 50 in each group. The animals were housed in sets of five in cages with solid bottoms and fed Purina Laboratory Chow until the experimental diets were instituted.

The diets were composed of semi-purified known components and in general were prepared, stored, and fed as previously described (11). To achieve a desired level of dietary protein the proportions of casein and cornstarch were reciprocally varied. Constituents other than protein and cornstarch—fat, salt mixture, and vitamin supplements—were present in the same amounts in the several diets of an experiment. The diets contained 2 per cent of gelatin as a binder, except for one ration in which it was present at the level of 11 per cent (2 per cent as binder plus 9 per cent as supplement). When DL-methionine and L-cystine were employed to increase the proportion of sulfur-containing amino acids of a low protein diet to that of a higher protein diet, they were added in excess of the calculated amounts (primarily to obviate the possible effects of more rapid absorption of the supplementary amino acids in comparison with those of the protein). The caloric values of the rations were computed from data supplied by the manufacturers; the dietary content of protein and of sulfur-containing amino acids was calculated from the following data: casein—93 per cent protein, 3.4 per cent methionine, and 0.3 per cent cystine; gelatin—88 per cent protein, 1.0 per cent methionine, and 0.06 per cent cystine.

Each week the mice were inspected and weighed. The incidence of hepatomas was determined at autopsy when the mice were 13–14 months of age. The tumors were recognized grossly; a considerable number was examined microscopically, including the few questionable lesions.

In one experiment analyses were performed on the livers and hepatomas of a number of animals. Protein-nitrogen was determined by a micro-Kjeldahl method (7) as the nitrogen insoluble in 5 per cent trichloroacetic acid; glycogen was determined by the method of Good, Kramer, and Somogyi (1).

EXPERIMENTS

Experiment 1.—Five groups of approximately 50 mice, 18 weeks of age, were given the rations shown in Table 1. Groups CA 1, CA 2, and CA 3 were fed diets in which the casein contents were 9, 18, and 45 per cent, respectively. The rations for groups CA 4 and CA 5 were modifications of that given group CA 1. In ration CA 4, 9 per cent gelatin replaced 9 per cent cornstarch; thus, it contained approximately the same amount of protein as the diet for group CA 2, but an amount

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of sulfur-containing amino acids similar to that of the diet for group CA 1. The ration for group CA 5 was the same as that for group CA 1, except that it was supplemented with 0.9 per cent DL-methionine

TABLE 1

COMPOSITION OF THE DIETS EMPLOYED IN EXPERIMENT 1*

Group	Casein	Gelatin	DL-Methionine (per cent)	L-Cystine	Cornstarch†
CA 1	9	2			79
CA 2	18	2			70
CA 3	45	2			43
CA 4	9	2+9			70
CA 5	9	2	0.9	0.1	78

* Mice were fed and consumed 10.2 Calories daily.

† Each diet also contained 4 per cent salt mixture (13), 5 per cent partially hydrogenated cottonseed oil (Kremax), 1 per cent rice bran extract (Vitab), and the following vitamin supplements per mouse daily: 20 USP units of A, 2 USP units of D, 0.8 mg. of E (the A, D, and E were in 0.005 ml. cottonseed oil); riboflavin, 30 μg.; thiamine hydrochloride, 60 μg.; pyridoxine hydrochloride, 60 μg.; Ca pantothenate, 180 μg.; niacin, 300 μg.; folic acid, 12 μg.; biotin, 0.3 μg.; *p*-aminobenzoic acid, 120 μg.; inositol, 1.5 mg.; and choline chloride, 15 mg.

group fed 9 per cent casein (CA 1) developed significantly fewer hepatomas than the group fed the isocaloric ration containing 18 per cent casein (CA 2). The addition of methionine and cystine to the 9 per cent casein diet resulted in a hepatoma incidence equivalent to that of the group fed 18 per cent casein (CA 5 compared with CA 2). On the other hand, supplementing the 9 per cent casein ration with 9 per cent gelatin resulted in only a negligible increase in the incidence of hepatomas (CA 4 compared with CA 1).

In an earlier experiment (11), mice fed a diet containing 45 per cent casein had a lower incidence of hepatomas than those fed an 18 per cent casein diet; the mice on the 45 per cent casein ration ate less food and weighed less, and it was considered that these factors might be responsible for the decreased incidence of tumors. The results of the present experiment confirm this view in that the mice fed 45 per cent casein developed a somewhat higher incidence of tumors than those fed 18 per cent casein in isocaloric amounts. The

TABLE 2

EFFECT OF THE PROPORTION OF DIETARY PROTEIN AND SULFUR-CONTAINING AMINO ACIDS ON THE FORMATION OF SPONTANEOUS HEPATOMAS (EXPERIMENT 1)

	Diets*		Dietary content of				Body weight‡ level (gm.)		Mice with hepatomas	
	Casein	Gelatin		Methionine		No.†			Number	
Group	(per cent)		Protein§	(per cent)	Cystine	mice	Range	Mean		Per cent
CA 1	9	2	10.1	0.33	0.03	52	21–30	27	1	2
CA 2	18	2	18.5	0.63	0.05	56	25–36	30	18	32
CA 3	45	2	43.6	1.55	0.14	48	24–37	31	21	44
CA 4	9	2+9	18.1	0.42	0.03	54	21–32	28	5	9
CA 5	9‖	2	11.1	1.23	0.13	44	22–34	29	13	30

* Complete diets given in Table 1; groups were given and consumed equicaloric amounts.

† Number of mice when sacrificed at 14 months of age.

‡ Body weight levels of individual animals from 10 months of age to the end of the experiment.

§ Moisture-free protein; in CA 5 the value includes the added amino acids.

‖ Supplemented with 0.9 per cent DL-methionine and 0.1 per cent L-cystine.

and 0.1 per cent L-cystine; i.e., it had approximately the same amount of protein as the diet fed group CA 1 but an amount of sulfur-containing amino acids greater than that present in ration CA 2. The proportions of protein, methionine, and cystine in the five diets are given in Table 2.

The diets were fed at a restricted level (10.2 Calories daily) to ensure complete consumption of the daily rations and thereby equicaloric intakes among the several groups. The body weights of the mice changed from an initial average value of 31 gm. to the levels shown in Table 2; given are the ranges of individual body weights of the mice and the mean values for the several groups during the last 4 months of the experiment.

When the mice were 14 months old, they were killed and examined for hepatomas. The results (Table 2) may be summarized as follows: The augmentation was not of a statistically significant magnitude, and it is concluded that increasing the proportion of dietary casein from 18 to 45 per cent results in no great effect on the incidence of spontaneously occurring hepatomas.

The data of this study suggest that the proportion of dietary sulfur-containing amino acids may be a critical factor in the rate of formation of spontaneous hepatomas in the mouse. The comparisons can be made from the values in Table 2.

It was noted that the incidence of hepatomas among the 5 groups was directly correlated with the mean body weights; these varied, even though the groups had been fed and had consumed equicaloric rations. However, the differences in body weight were probably not sufficient to account for more than a small part of the differences in tumor formation (12). Nevertheless, in

the following experiment undertaken to confirm the significance of sulfur-containing amino acids, the mean body weights of the groups were kept approximately equal by adjustment of the food intake.

Experiment 2.—Three groups of 50 mice, 24 weeks of age, were fed diets similar in composition to those for groups CA 1, CA 2, and CA 5 of Experiment 1. Group CH 1 was fed *ad libitum* a ration containing 9 per cent casein, and group CH 2 a ration with 19 per cent casein. Group CH 3 was given a ration with 9 per cent casein and 0.6 per cent DL-methionine and 0.03 per cent L-cystine; diet CH 3, therefore, contained approximately the same amount of protein as diet CH 1, but higher proportions of methionine and cystine than diet CH 2. At the beginning of the study the individual animals ranged from 28 to 44 gm., each group averaging 35 gm. The mice were weighed once a week, and, when necessary, the amounts of rations for groups CH 2 and CH 3 were adjusted to achieve the same mean body weight as that of the *ad libitum*-fed group CH 1; the range of food intake and the mean for each group are given in Table 3. As it happened, the groups maintained equal average body weights between 34 and 36 gm. during the entire experiment. The investigation proceeded smoothly, and the mice were examined for hepatomas when they were 13–14 months of age.

TABLE 3

DEPENDENCE OF HEPATOMA FORMATION ON THE PROPORTION OF SULFUR-CONTAINING AMINO ACIDS (EXPERIMENT 2)

Group	Dietary* casein, Per cent	Daily food† intake (gm.) Range	Mean	Number‡ of mice	Mice with hepatomas Number	Per cent
CH 1	9	3.8–4.0	3.9	49	7	14
CH 2	19	2.8–3.6	3.2	50	22	44
CH 3	9§	2.9–3.8	3.4	48	22	46

* See text for description of diets.

† Amounts of ration were adjusted weekly to maintain equal average body weights, which varied from 34 to 36 gm. during the experiment.

‡ Number of mice when sacrificed at 13–14 months of age.

§ Supplemented with 0.6 per cent DL-methionine and 0.03 per cent L-cystine.

The results (Table 3) confirm the data obtained in the previous experiments: as compared to the ration containing 9 per cent (CH 1), the augmenting effect on the incidence of hepatomas of the ration with 19 per cent casein (CH 2) was entirely duplicated by the one containing 9 per cent casein supplemented with sulfur-amino acids (CH 3). Furthermore, the incidence was not related to the mean food intake, and the results of both experiments indicate that the effect obtained was not due to differences in caloric intake or body weight.

At the end of the experiment the mice were anesthetized with nembutal so that the livers, in addition to being inspected for hepatomas, could be analyzed for protein and glycogen. Approximately half the mice were sacrificed before fasting, the others after being deprived of food for 24 hours (during which time they lost approximately 15 per cent of their body weight). Whenever a hepatoma more than 6 mm. in diameter was found, both the hepatoma and nearby normal liver were analyzed, the samples for the latter being taken first. The livers of a number of mice without tumors were also studied to provide reference values.

Among the mice with hepatomas, the normal portions of the livers were similar in protein and glycogen content to those of the nontumor-bearing mice. However, the hepatomas invariably contained a higher percentage of glycogen and, except in one case, a lower level of protein than the adjacent normal liver. This is shown by the ratios of the glycogen and protein concentrations in the hepatoma to the concentrations in the liver given in Table 4. In both normal hepatic tissue and hepatoma a 24-hour fast resulted in a decrease in glycogen and an increase in protein, but comparison of the ratios for mice deprived of food with those for mice not deprived of food for 24 hours suggests that during fasting the hepatoma did not lose as great a proportion of glycogen as the normal liver, nor did the protein concentration increase as rapidly.

TABLE 4

GLYCOGEN AND PROTEIN CONTENT OF HEPATOMA AND NORMAL TISSUE OF SAME LIVER

	Ratio of concentration in hepatoma to that in normal tissue: Glycogen	Protein
Full-fed mice	(1.03–2.71)* 1.73±0.22	(0.72–1.02) 0.84±0.053
Mice on fast	(1.43–3.42) 2.48±0.23	(0.64–0.79) 0.72±0.026

* The figures in parentheses indicate the range of individual values, nine mice in each group. The figures not in parentheses are the means and standard errors of the means.

DISCUSSION

The present studies confirm our previous report (11) that a decrease in the proportion of dietary casein from approximately 18 per cent to 9 per cent results in a striking retardation in the rate of formation of spontaneously occurring benign hepatomas in C3H mice. The inhibition is due to a deficiency of sulfur-containing amino acids in the 9 per cent casein diet and not to the difference in the proportion of total protein. This is indicated by the finding that the supplementation of the 9 per cent casein diet with methionine and cystine

resulted in a hepatoma incidence equal to that produced by a diet containing 18 per cent casein; while only a minimal effect (of a magnitude compatible with its methionine content) followed supplementation with 9 per cent gelatin. Furthermore, the design and results of the experiments show that the differences in hepatoma formation were not dependent on inequalities in caloric intake and body weight.

The data also suggest that increasing the portion of dietary casein from 18 per cent to 45 per cent does not greatly affect the incidence of hepatomas. It is, therefore, probable that the rate of formation of these tumors is dependent on an optimal or critical amount of nutritionally adequate protein, and that increases above this amount have little influence upon tumor formation.

With regard to other tumors of the mouse, a reduction of dietary casein from 18 per cent to 9 per cent had virtually no inhibitory action upon the formation of spontaneously occurring mammary carcinomas or induced skin tumors (11). It is possible that decreasing the casein content to a still lower proportion and the employment of moderate carcinogenic activity would result in effects comparable to those seen with the spontaneous hepatoma. The influence of the proportion of dietary protein on the formation of induced liver tumors in the rat is modified by other features of the regimen, such as the levels of riboflavin (5) and pyridoxine (8) and the carcinogen employed (3). While there have been occasional reports that the proportion of protein had little or no effect (4, 9), other experiments indicate that decreases in casein content from 24 per cent or more to 10 or 12 per cent augmented the formation of tumors induced by *p*-dimethylaminoazobenzene (2, 10). However, it is significant that, in contrast to the inhibitory action on the spontaneous hepatoma of the mouse, such a decrease in dietary protein has never been reported to result in a decreased incidence of induced hepatic tumors in rats.

Other investigators have examined the influence of the level of dietary sulfur-containing amino acids on the formation of various types of tumors of the mouse and rat (2, 4, 6, 14, 15, 16, 17); the findings vary with the kind of tumor, and in some instances interpretation of the results is obscured by the concurrent effects of caloric intake and body weight. Nevertheless, the experiments of White and associates (16, 17) suggest that a diet low in cystine inhibits the formation of methylcholanthrene-induced leukemia in mice; they interpret the effect as not being associated with the properties of cystine as an amino acid essential for growth.

The fact that supplementing a 9 per cent casein diet with methionine and cystine resulted in an incidence of hepatomas equal to that found in mice on an 18 per cent casein diet does not mean that sulfur-containing amino acids act specifically in the carcinogenic process. There remains another and more probable explanation centering about the likelihood that the genesis of hepatomas is influenced by the proportion of nutritionally adequate or "balanced" protein. Because casein is relatively deficient in methionine, the 9 per cent casein ration actually contained less than 9 per cent "balanced" protein. Supplementation with 9 per cent gelatin increased the total amount of protein as measured by amino nitrogen but only slightly affected the amount of nutritionally adequate protein. On the other hand, adding the sulfur-containing amino acids had little effect on the total protein but definitely augmented the level of nutritionally adequate protein. Further experimentation is necessary to demonstrate the exact factors responsible for the observed effects on hepatoma formation.

SUMMARY

C3H male mice ingesting a diet containing 9 per cent casein as the principal source of protein developed a significantly lower incidence of hepatomas than mice on a diet containing 18 per cent casein. This occurred whether the daily rations were isocaloric or were adjusted to maintain equivalent body weights. Changing the proportion of dietary casein from 18 to 45 per cent had no noteworthy effect on the incidence of hepatomas.

The amount of dietary protein per se was not the factor responsible for the striking difference in the rate of formation of hepatomas between mice on 9 per cent and those on 18 per cent casein diets. Adding 9 per cent gelatin to the 9 per cent casein ration had little effect. On the other hand, supplementing the 9 per cent casein ration with methionine and cystine increased the incidence of hepatomas to that of mice on an 18 per cent casein diet. Thus, the reduced incidence of hepatomas in the mice on the 9 per cent casein rations was a consequence of inadequate amounts of dietary sulfur-containing amino acids. The supplements of these amino acids may have acted directly in the carcinogenic process or, more likely, produced their effect by augmenting the nutritional adequacy of the dietary protein.

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The Effect of Complete Ablation of Thyroid Tissue by Radioactive Iodine on the Survival of Tumor-bearing Mice*

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Recent work in this laboratory with a radioactive iodine (I^{131}) derivative of Nile blue 2B (an oxazine dye) has demonstrated that this radioactive dye has a significant effect in prolonging the life of mice bearing transplanted tumors (12). It was found, in this study, that considerable radioactivity was present in the thyroid glands of animals to which this dye was administered. Presumably, in the living animal some of the organically bound iodine is split from the dye molecule, and the resulting iodide is concentrated in the thyroid gland. Thus, it is possible that some thyroid tissue might have been destroyed by the radioactive iodine and that this might have had some effect on the survival of the tumor-bearing mice. Accordingly, the present study was undertaken to evaluate the effect of ablation of thyroid tissue by radioactive iodine on the survival of tumor-bearing mice.

Previous studies on the effect of surgical removal of the thyroid gland on the growth of tumors have yielded variable results. It has been reported that surgical thyroidectomy has no effect on growth of tumors (1), causes regression of tumors (9, 13), causes resistance to induction of tumors (11), and accelerates growth of tumors (7, 8). Surgical thyroidectomy is frequently accompanied by unintentional removal of the parathyroid glands. (Operative mortality in the experimental animal is generally high.)

Gorbman (5) showed that complete destruction of thyroid tissue without concomitant complete destruction of parathyroid tissue could be achieved by the administration of radioactive iodine (I^{131}) to adult mice. Similar studies in the adult and newborn rat (3, 4) have shown that thyroidectomy without parathyroidectomy can be achieved with radioactive iodine.

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EXPERIMENTAL

Inbred mice of the C3H strain, each weighing approximately 25 gm., were used. The mice were allowed Purina Laboratory Chow and tap water *ad libitum*. Either of two fibrosarcomas (Nos. 13 and 14), which had been originally induced in mice of this strain with methylcholanthrene, was used. These tumors have been transplanted through many generations with a zero incidence of spontaneous regression. The tumors were implanted by subcutaneous injection into the right axillary region. Radioactive iodine (carrier-free I^{131} obtained from the Oak Ridge Laboratories of the Atomic Energy Commission) was administered by a single subcutaneous injection at a site distant from the tumor.

The experiments were divided into two groups. In the first group, radioactive iodine was administered after the tumors had grown to appreciable size. In the second group, radioactive iodine was administered either 34 days or 103 days prior to implantation of the tumor. In each case the number of days that elapsed between implantation of the tumor and death of the mouse was noted and recorded as survival time.

In one experiment the mice were housed individually in separate cages to eliminate any possible effect of external radiation from one mouse to another. In the other experiments each group of mice was housed in a single large cage.

The dosage of radioactive iodine was 250 μc. per mouse, except in one experiment in which the dosage was 210 μc. Gorbman (5) has reported that a dose of 200 μc. is sufficient to destroy all thyroid tissue in the mouse.

RESULTS

Table 1 shows the survival times of mice which received 210 μc. of radioactive iodine 8 days after implantation of a fibrosarcoma (tumor No. 13) and of control mice which bore the same tumor and were treated in identical fashion except that

they received no radioactive iodine. The average weight of the mice at the time of administration of radioactive iodine was 22 gm. Seventeen days later the average weight was 26 gm. for the treated mice and 28 gm. for the control mice. The mice

TABLE 1

210 MICROCURIES I^{131} ADMINISTERED 8 DAYS AFTER IMPLANTATION OF TUMOR (NO. 13)

DAYS SURVIVED	
Controls (No I^{131})	Treated
27	18
27	18
28	29
31	31
31	31
35	31
36	32
37	43
42	43
42	43
42	44
44	44
44	44
45	53
46	
AV. 37	AV. 36

were normally active until shortly before death, at which time all had very large tumors. The only suggestion of apparent difference between the treated and control mice was that the treated mice seemed to consume a little less food than the controls.

Table 2 shows the survival times of mice which received 250 μc. of radioactive iodine either 12 days after or 34 or 103 days prior to implantation of a fibrosarcoma (tumor No. 14). The average body weight at the time of administration of radioactive iodine was 24 gm. for the control mice and 25 gm. for the mice which received radioactive iodine 12 days after implantation of tumors. Twenty-one days later the average weights were 27 gm. for both of these groups. Again, there was no noticeable difference between the control and treated mice except that in the group which received radioactive iodine 103 days prior to tumor implantation there was moderate loss of hair pigment on the ventral aspect of the body, especially around the area of tumor implantation.

There was no noticeable difference in the rate of growth of the tumors, and in all mice the tumors were very large at the time of death. In two groups, rough measurements of the tumor size of control and treated mice at 21 days and 28 days after implantation of tumors, showed that the average tumor volume was approximately the same for both control and treated mice.

Gorbman had shown (5) by serial section of the neck region that doses of radioactive iodine of the order of magnitude used in these experiments produced complete destruction of thyroid tissue in the mouse. In the present study the neck region of a mouse treated with radioactive iodine was serially sectioned. Microscopical examination of these sections showed only remnants of disrupted thyroid follicles and no thyroid tissue which appeared capable of function was seen. Other contiguous tissues were normal. In a number of other treated mice, sections of the thyroid region likewise showed no thyroid tissue capable of function. The thyroid glands of the control mice were grossly and microscopically normal.

TABLE 2

250 MICROCURIES I^{131} ADMINISTERED BEFORE OR AFTER IMPLANTATION OF TUMOR (NO. 14)

	DAYS SURVIVED		
Controls (No I^{131})	I^{131} 12 days after implantation of tumor	I^{131} 34 days prior to implantation of tumor	I^{131} 103 days prior to implantation of tumor
25	29	30	31
26	32	32	34
33	39	32	36
35	39	33	37
39	43	34	38
39	46	34	40
39	47	35	40
40	47	35	41
40	48	36	41
43	53	37	43
44	53	37	44
47	53	37	46
48	53	39	47
49	53	39	48
50	AV. 45	39	49
AV. 40		40	50
		40	AV. 42
		41	
		41	
		41	
		42	
		43	
		AV. 37	

DISCUSSION

It is apparent from the data of Tables 1 and 2 that there is no significant difference (P is greater than 0.05 for all groups) in survival time between the control animals and those whose thyroid glands were ablated either before or after implantation of the tumor. The results demonstrate reasonably well that, under the conditions of these experiments, ablation of thyroid tissue by injections of radioactive iodine has no appreciable effect on the growth of transplanted fibrosarcomas or on the survival of the host animal.

Drabkin (2) suggests that thyroid activity may be mediated via cytochrome c, and he has shown that there is a reduction in cytochrome c in all tis-

sues of the hypothyroid animal. Meyer, McTiernan, and Aub (10) found that alteration of thyroid activity (by administration of thyroxine) had no effect on either aerobic or anaerobic glycolysis of tumor tissue.

Neoplastic tissue is known (6) to be quite low in cytochrome c, and it might be expected that alterations in thyroid function would have less effect on tumor metabolism than on other tissues of the animal. Furthermore, tumor tissue has a greater ability for satisfying its metabolic requirements by glycolytic processes than does normal tissue. As a result of all this, little or no stress on the functioning of the tumor might be expected from the depression of oxidative processes such as results from thyroid ablation.

SUMMARY

1. Ablation of thyroid tissue in mice bearing transplanted fibrosarcomas has been carried out by administration of radioactive iodine (I^{131}). This ablation was done both prior to and after implantation of the tumors.

2. The average rate of growth of the tumors and the average period of survival of the mice in which thyroid tissue had been ablated were not significantly different from those of untreated tumor-bearing mice.

3. It is concluded that thyroid ablation has no significant effect on tumor growth.

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The Development of Mammary Cancer in Castrate A Strain Male Mice Bearing Ovarian Grafts*

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The early studies of Murray (11) suggested that male mice that had been castrated and transplanted with ovaries developed mammary cancer with approximately the same frequency as did virgin female mice of the same strain. Later, Loeb *et al.* (10), in a preliminary report in which no data were presented, stated that "after transplantation of ovaries into castrate male mice, the cancer rate and proliferative as well as secretory activities of the mammary gland were greater than after transplantation of these organs into normal female mice." With male mice of a hybrid cross, in which a high percentage of the virgin female mice develop mammary cancer, it was observed in this laboratory that castrate males bearing ovarian grafts developed mammary cancer with great frequence and at a significantly younger average age than did the virgin female mice of this particular cross (8). The present investigation was undertaken to study the rate and time of mammary carcinogenesis in male mice of a stock in which the virgin female mice have a low incidence of cancer, although breeding females have a high incidence.

MATERIALS AND METHODS

Male mice of the A stock maintained in this laboratory were castrated shortly after being weaned (4–6 weeks of age), and at the same time two ovaries that had been freed from the encompassing ovarian sac were implanted deep in the axillary tissue. The animals were then housed in wooden boxes, fed Purina Fox Chow *ad libitum*, and inspected once a week for the appearance of subcutaneous tumors. All animals developing palpable subcutaneous tumors were examined at autopsy, and the tumor mass as well as the ovarian transplant was prepared for microscopic examination. In addition, nine of the nontumorous mice were sacrificed for histological examination when they appeared to be approaching *exitus* and the development of mammary cancer appeared unlikely.

Approximately half the animals received ovaries from donor A females, while the others received transplants of ovaries from donor Ax females. The Ax strain was originated in 1934 (3) by foster nursing one litter of strain A mice of the 41st inbred generation on a female of the CBA strain (designated as the X stock) that lacked the milk agent. Since that time, the A and Ax stocks have been maintained entirely independent of one another by brother to sister matings. The transplantations reported in this study were carried out between October, 1945, and March, 1948, at which time the A strain mice, both donors and recipients, were of the F73 through F80 generations, while the Ax donor animals were of the F25 through F32 generations (the original fostered litter has been considered as the F1 generation). It should, therefore, be stressed that there had been no interchange of genetic material between these two separate lines of mice for from 57 to 71 generations prior to their use in this experiment.

RESULTS

Tumor development.—Approximately the same percentage of males bearing Ax ovaries developed mammary cancer as did males bearing A ovaries, so that, regarding tumor development, these two groups of animals may be considered as one (Table 1). The incidence of tumor development in these castrate male mice bearing ovarian grafts is significantly higher than the 4 per cent incidence noted in virgin female mice of this strain maintained in this laboratory (4) or the 5 per cent incidence reported in a small group of ovariectomized A mice bearing subcutaneous ovarian grafts (7). However, it is well below the 80.5 per cent incidence observed in breeding A strain mice during

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this period, and the average age of tumor development is considerably (approximately 3 months) later.[1]

Ovarian histology.—In all mice seen at autopsy (22 bearing A ovaries and 15 bearing Ax ovaries), viable ovarian grafts were found. Among the younger animals follicles were numerous, a few small corpora lutea were seen, and the interstitial tissue was generally hypertrophied and contained large, agranular interstitial cells. In short, the histology of these ovaries was essentially the same as that previously described (8) for hybrid ovaries transplanted to castrate F1 hybrid males (A × Z), in spite of the fact that the ovarian histology of normal female mice of A strain differs considerably from that of the normal AZF1 females (7).

It is also of interest to record that in four of the ovarian transplants studied histologically (three from A donors and one from an Ax donor) there was an overgrowth of granulosa cells to form typical, small granulosa-cell tumors similar to those described previously in both male and female animals of the A × Z cross bearing transplanted ovaries (8). These occurred in animals 16–20 months of age, and concurrent mammary tumors were present in three of the four animals. No attempt was made to evaluate the hormone production of these ovarian tumors.

Mammary gland histology.—The mammae of sixteen animals (thirteen tumorous and three nontumorous) were studied histologically, by use of both the whole mount and section technics. The degree and type of development varied considerably from one animal to another and even within different glands of the same animal. This variation was much greater than that seen in virgin or breeding female mice of this stock. In general, it can be said that the glands of these male mice exhibited greater development than do those of virgin female mice of this stock. Lateral budding, which is rather minimal in the glands of A strain virgin females (6) (Fig. 1), was prominent in most of the glands studied and was frequently very extensive (Fig. 2). In the glands of several of the animals there were areas of normal lobular development resembling that seen in female mice late in pregnancy or on the first *post partum* day. These areas varied considerably in size, from small clusters of alveolae to areas measuring several mm. in diameter, but the histology of all was essentially the same (Figs. 3, 4). The alveolar lumina were rather large and generally contained secretion; many of the epithelial cells had secretory vacuoles within their cytoplasm; and there was less connective tissue about the alveolae than is usually encountered in the typical precancerous hyperplastic nodules seen in the resting glands of female mice of high tumor strains (6). In addition, however, typical precancerous hyperplastic nodules, which are but rarely encountered in the glands of virgin A strain mice, were moderately frequent in these male glands.

In several instances, the larger ducts were moderately to greatly distended with secretion (Fig. 5). This was most marked in one gland in which there were no lateral buds or "alveolar" formation, so that at least in this instance the secretion was formed by the duct epithelium itself. In general, the pattern of duct development seen in the glands of these male mice was rather similar to that encountered in female mice (Fig. 6). In a few instances, however, a peculiar and extensive development of very fine ducts was observed. This differed from the localized, precancerous "fine duct nodule" (5) occasionally seen in the glands of some stocks of female mice, in that in these males the small ducts occupied a large part of the fat pad and presented no particular orientation as is the case of the "nodules" seen in female glands. All in all, the glands of these male mice were considerably more developed than are the glands of virgin female mice of this stock, and in certain areas their development simulated that seen in the gland of female mice during the latter part of pregnancy. In addition, they presented some atypical features not encountered in the mammae of normal female mice.

TABLE 1

THE DEVELOPMENT OF MAMMARY CANCER IN CASTRATE MALE MICE OF THE A STRAIN BEARING OVARIAN GRAFTS

Strain of donor female	No. tumorous/ no. animals	Per cent developing cancer	Average age (months)	
			Cancer development	Noncancer deaths
A	16/38	42.1	14.6	16.6
Ax	12/32	37.5	15.1	18.2
TOTAL	28/70	40.0	14.8	17.3

DISCUSSION

From more recent evidence obtained in this and other laboratories, it would appear that castrated male mice bearing ovarian grafts develop mammary cancer with greater frequency than do virgin female mice of the same strain. This increased frequency of cancer development, as well as the more extensive development of the mammary glands in these male mice, may be, at least in part, the result of the essentially non-cycling male pituitary. Thus, when both vaginal fragments and ovarian tissue were transplanted to castrate male mice, the

[1] J. J. Bittner, unpublished data.

vaginal epithelium was found to be continuously cornified, suggesting a rather constant stimulation of the ovarian graft by the male hypophysis (8). In view of this, it is interesting to note that Loeb *et al.* (10) and Silberberg and Silberberg (12) all have observed that if additional pituitary tissue is transplanted to castrate male mice along with the ovarian tissue, a higher incidence of mammary cancer results than if ovarian tissue alone is transplanted. The rather pronounced tendency for the mammae of castrated, ovarian-transplanted male mice to have secretion in their ducts, as well as areas of normal alveolar development similar to that seen in late pregnancy, might also suggest an increased production of pituitary lactogenic hormone by the male pituitary.

The evidence obtained in these experiments for the genetic stability of an inbred stock of mice is also rather remarkable. Work with the transplantation of both normal (2) and malignant tissue (1, 9) has shown that the successful growth or maintenance of transplanted tissue is governed by genetic principles and that, in general, eight to fourteen factors are involved in the maintenance of grafted normal tissue. The fact that ovarian tissue of the Ax stock could be 100 per cent successfully grafted into the subcutaneous tissue of A strain mice when there had been no interchange of genetic material between these lines for from 57 to 71 generations would seem to indicate a great deal of genetic stability, once a genetically pure strain has been obtained.

SUMMARY

Forty per cent of a group of castrate A strain male mice bearing subcutaneous ovarian grafts developed mammary carcinoma at an average age of 14.8 months. Only 4 per cent of virgin female mice of this strain developed mammary cancer at an average age of 15.0 months, while during the period of this experiment 80.5 per cent of breeding females were afflicted at an average age of 11.7 months. It is, therefore, evident from these and other experiments that the tendency for castrate male mice bearing transplanted ovaries to have mammary cancer is intermediate between that noted in virgin and in breeding mice of the same stock. A study of the mammae of these male mice revealed them to be considerably more developed than those of virgin A strain mice, in that they possessed much more lateral budding, frequently contained considerable secretion in their ducts, and had areas of normal-appearing alveolae as well as typical areas of precancerous nodular hyperplasia.

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Fig. 1.—A typical area of a whole mount preparation of the mammary gland of a virgin A strain female mouse 356 days of age. Note the paucity of lateral buds protruding from the smaller ducts.

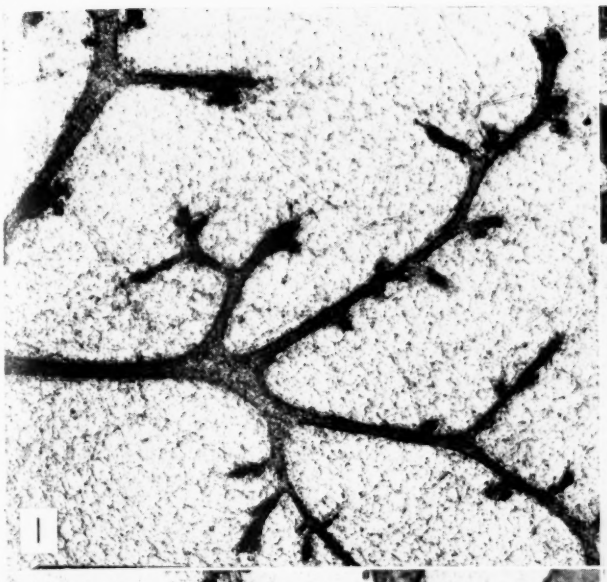
Fig. 2.—An area of a whole mount preparation of a castrate A strain male mouse 303 days of age that was grafted with ovaries when 1 month of age and had a mammary gland carcinoma when sacrificed. The amount of lateral budding shown here is about average for the group of male mice studied, although there was a great degree of variation from animal to animal.

Fig. 3.—An area of a whole mount preparation of a castrate A strain male bearing grafted ovaries. There are two small clusters of normal-appearing alveolae in the field, as well as frequent lateral buds along the smaller ducts.

Fig. 4.—A section of a small cluster of normal alveolae similar to those seen in Figure 3. Note the luminal secretion, the intracytoplasmic secretion vacuoles, and the sparseness of the surrounding collagenous connective tissue.

Fig. 5.—A view at low magnification of a section of a mammary gland of a castrate A strain male bearing ovarian grafts. In this animal the larger ducts are distended with secretion and there is a considerable alveolar development in one area.

Fig. 6.—A view at low magnification of a whole mount preparation of the mammary gland of a castrate A strain male bearing grafted ovaries. The extent and general architecture of the duct development is similar to that seen in virgin female mice of this strain; however, the more extensive lateral budding is evident even at this low magnification, and there are several small areas of precancerous alveolar hyperplasia visible.



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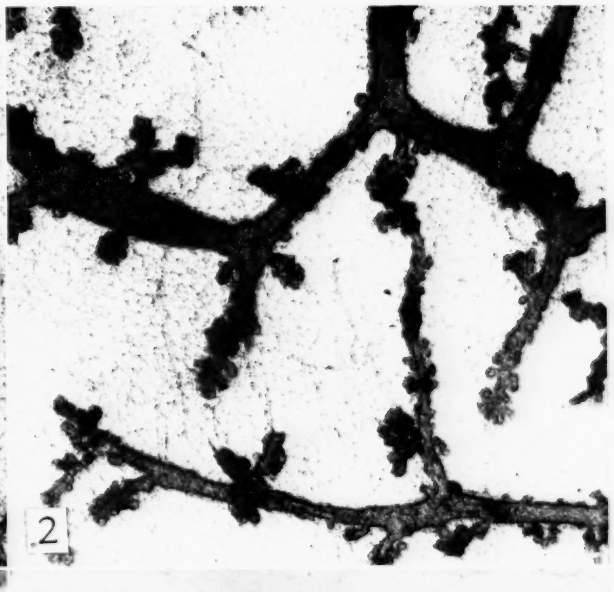

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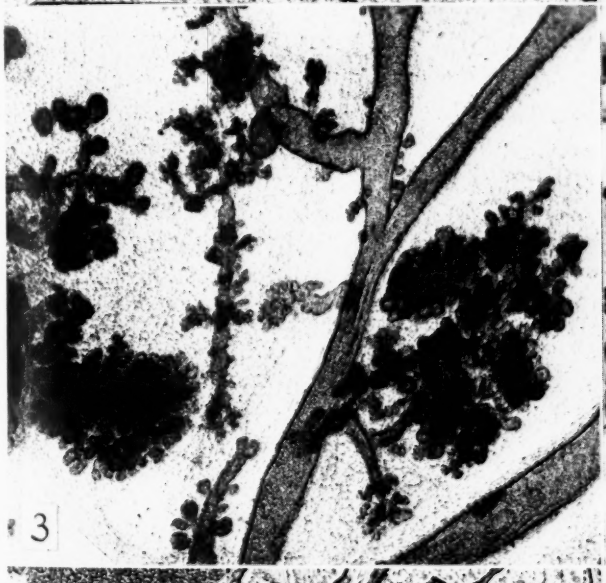

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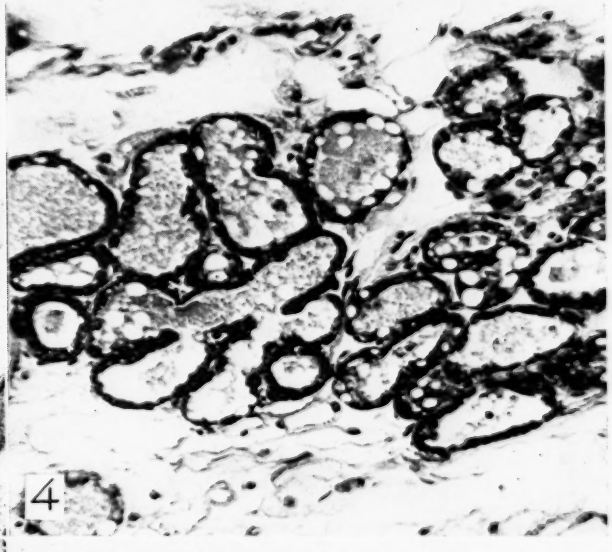

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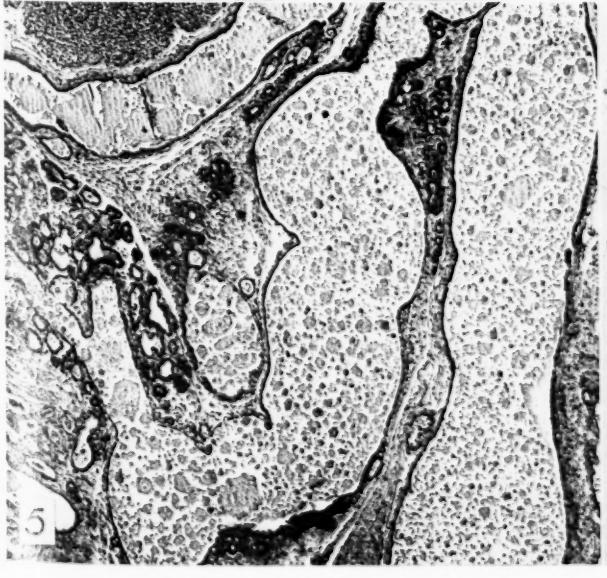

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6

Study of the Tryptophan-Acid and Iodoacetate Index Tests in Patients with Malignancy*†

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In an earlier paper (7) it was shown that the tryptophan-acid test described by Seibert (9) was only 66 per cent correct in diagnosing the presence of malignancy when used as a test for malignancy by us. Approximately the same percentage in the use of the iodoacetate index described by Huggins (4)[1] has been reported (2, 3, 6). The work reported below confirms, with a larger series of cases, the finding that neither test is a specific diagnostic test for cancer. In addition, it shows that, although the simultaneous use of both tests increases somewhat the per cent accuracy of diagnosis, the use of both tests together is likewise not sufficiently specific for the accurate diagnosis of malignancy. Seibert thought the substance producing the tryptophan-acid reaction might be the result of tissue breakdown. Studies of the tests on patients after operation, when tissue destruction occurs (1, 5, 8), were inconclusive.

OBSERVATIONS

Both the tryptophan-acid and iodoacetate index tests have been done on 1,328 persons, divided into the following groups:

A. *Healthy group* (224 individuals).—These were people who came to the Cancer Prevention Clinic for complete physical examination and were certified by the examining physician to be without evidence of any disease.

B-1. *Nonmalignant disease group—not hospitalized* (726 individuals).—Those who came to the Cancer Prevention Clinic for complete physical examination and were found to have various diseases (exclusive of cancer), such as hypertension, chronic cystic mastitis, sinusitis, obesity, menopausal symptoms, chronic cholecystitis, spastic colitis, bronchial asthma, benign hypertrophy, hypothyroidism, etc. None were hospitalized.

B-2. *Nonmalignant disease group—hospitalized* (137 individuals).—Those who had a variety of diseases, such as chronic infectious mononucleosis, regional enteritis, rectal polyp, fibroid of uterus, duodenal ulcer, calculous cholecystitis, gastric ulcer, chronic hepatitis, etc. All were hospitalized.

C. *Proved malignancies* (241 individuals).—These were patients on the medical and surgical wards of the hospital of the University of Pennsylvania. The samples of blood for analysis were taken pre-operatively or before x-ray or other therapy was instituted.

Group A, the healthy group, was used as a basis for establishing "normal" values in both tests. As can be seen in Chart 1, the mean value for all the healthy patients in the tryptophan-acid test was 50.3 (Klett colorimeter reading). Twice the standard deviation (σ) gave an upper limit of 73.5. We have therefore considered that for the tryptophan-acid test values of 74 or over were abnormal.

Similarly in the iodoacetate index test the mean value was 10.0, and twice the standard deviation gave a lower limit of 8.9. Anything below 8.9 was considered abnormal (Chart 2). Using these values as our criteria for a normal test, we found that thirteen of the healthy group of 224 had an abnormal tryptophan-acid test (5.8 per cent) and that six of the 224 had an abnormal iodoacetate index (2.6 per cent).

Mean values of the two tests for the four groups of patients are shown in Charts 1 and 2. Although the means for the two nonmalignant groups and for the malignant group differ significantly from the mean of the healthy or normal group in both tests, the distribution curves indicate that there is considerable overlapping between the groups.

In the nonmalignant group B-1 there were 67

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[1] The method used in this work to determine the iodoacetate index differed from that described by Huggins, Miller, and Jensen in that a commercial preparation of sodium iodoacetate was used instead of recrystallized iodoacetic acid.

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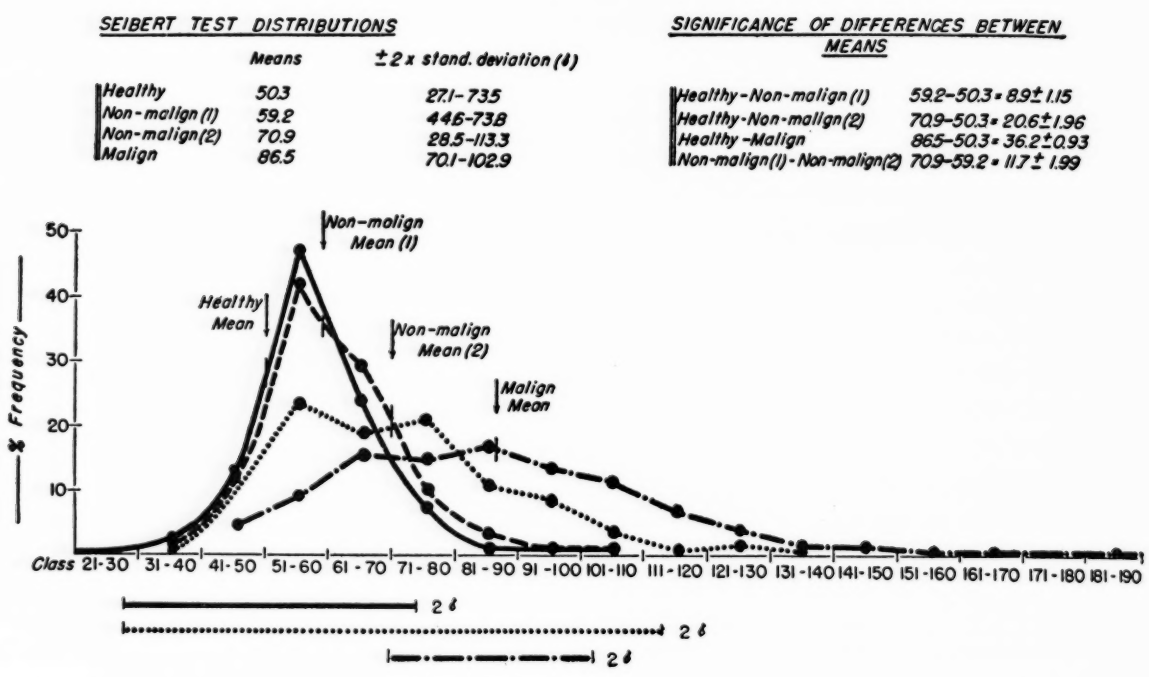

CHART 1.—Frequency distribution of tryptophan-acid values.

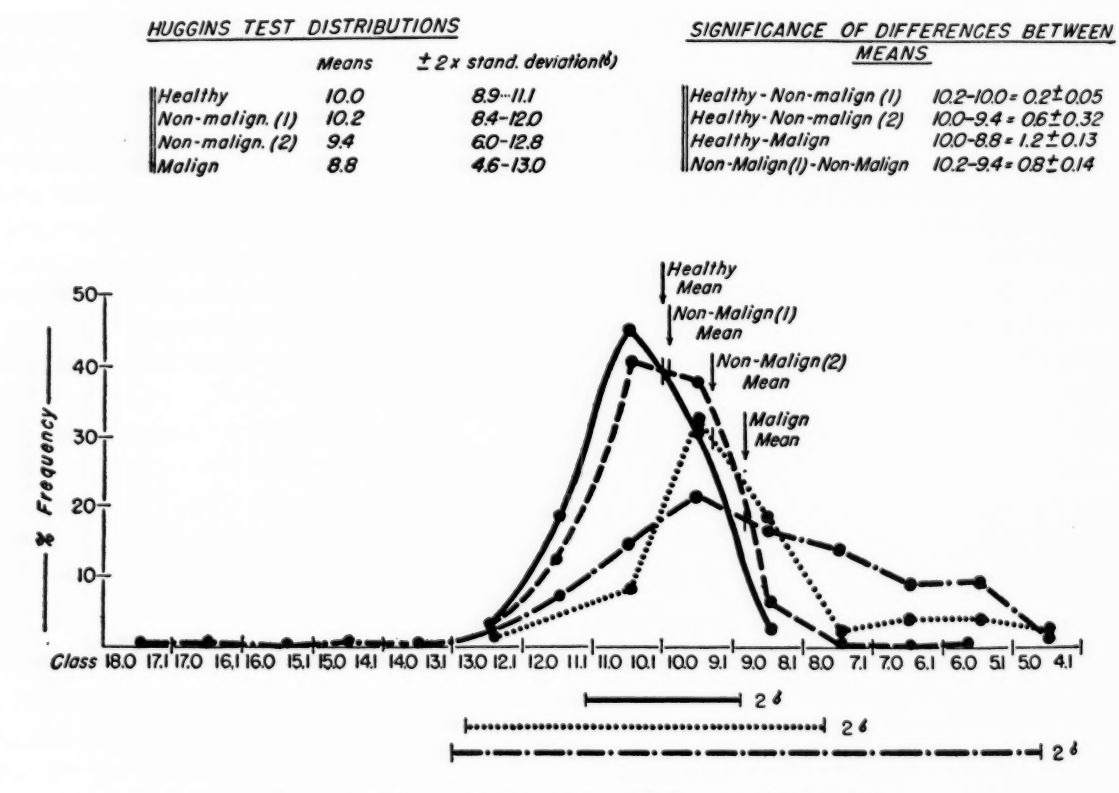

CHART 2.—Frequency distribution of iodoacetate-index values.

false positives by the tryptophan-acid test (9.2 per cent) and 37 false positives by the iodoacetate index test (5.0 per cent). In nonmalignant group B-2 there were 53 false positives by the tryptophan-acid test (38.6 per cent) and 40 by the iodoacetate index (29.1 per cent). Thus, there were fewer "false positives" with the iodoacetate index than with the tryptophan-acid test.

In the malignant group there were 85 normal values for the tryptophan-acid test (35.2 per cent) and 118 normal values for the iodoacetate index (48.9 per cent), showing that fewer "false negatives" are obtained with the tryptophan-acid test than with the iodoacetate index (Table 1).

TABLE 1

DISTRIBUTION OF POSITIVE AND NEGATIVE VALUES

		Tryptophan-acid		Iodoacetate index	
Groups	Total no.	Pos.	Neg.	Pos.	Neg.
A. Healthy	224	13 (5.8 per cent)	211	6 (2.6 per cent)	218
B-1. Nonmalignant	726	67 (9.2 per cent)	659	37 (5.0 per cent)	689
B-2. Nonmalignant	137	53 (38.6 per cent)	84	40 (29.1 per cent)	97
C. Malignant	241	156	85 (35.2 per cent)	123	118 (48.9 per cent)

Not all the 85 "normals" in the malignant group for the tryptophan-acid test were also "normal" for the iodoacetate index, but only a certain percentage of the group was normal for both tests. Similarly, not all positives in the tryptophan-acid test were also positive for the iodoacetate index. If, therefore, we take as our criterion for malignancy abnormal values in both tests, it is found (Table 2) that there were no persons in the

TABLE 2

ABNORMAL VALUES FOR BOTH TESTS SEPARATELY OR IN COMBINATION

	Total no.	Pos. tryptophan-acid only	Pos. iodoacetate index only	Pos. both tests
A. Healthy	224	13	6	0
B-1. Nonmalignant	726	59	31	6
B-2. Nonmalignant	137	34	21	19
C. Malignant	241	62	27	77

healthy group who had abnormal values for both tests, and that the number of people in the two nonmalignant groups having positive values for both tests was small. Thus, one reduces the number of "false positives," and the single positive values may be classified as "suspicious." In the malignant group there were 77 values (31.9 per cent) which were abnormal by both tests. In addition, there were 62 positives by the tryptophan-acid and 27 positives by the iodoacetate index test, which makes 89 values which may be regarded as "suspicious." This gives a total of 156 or 64.7 per cent.

As in the previous work (7), separation of the malignant group into "surgical" and "medical" divisions shows considerably more normal values in the surgical group than in the medical group. In the surgical group of 110, 37.3 per cent had normal tryptophan-acid values, and 51.8 per cent had normal iodoacetate indices. In the medical group of 131, 29.8 per cent had normal tryptophan-acid values and 48.9 per cent normal iodoacetate indices. Again the only explanation we can offer is that the members of the surgical group were possibly in an earlier stage of their disease.

Seventy-five patients with proved malignancy were studied both pre- and post-operatively. On 62 of the 75, the tryptophan-acid test was done pre-operatively and within 7 days post-operatively. On 64 of the 75, the iodoacetate index was done pre-operatively and within 7 days post-operatively.

Of the 62 on which the tryptophan-acid test was done before operation, the pre-operative value was below 74 (normal) in 20 and 74 or above (abnormal) in 42. Of those 20 which were normal before operation, 16 became abnormal after, and 4 remained normal. All those who had an abnormal test before operation remained abnormal after operation, although sometimes the value increased, sometimes it decreased.

Of the 64 on which the iodoacetate index was done, the pre-operative value was 8.9 or above (normal) in 28, and below 8.9 (abnormal) in 36. Of the 28 which were normal before operation, 16 became abnormal within 7 days post-operatively. Twelve remained normal.

Of those 36 who had an abnormal iodoacetate index before operation, 28 remained abnormal after operation, 8 became normal.

A group of 21 patients who were operated upon for diseases other than malignancy (polyps, benign ulcers, cystic mastitis, thyroid, bronchial cyst, cholecystitis, cystadenoma of pancreas, recto-vaginal fistula, etc.) also showed that the tests

could be either normal or abnormal pre-operatively and that in a majority (but not in all) the test was abnormal after operation.

The increase in number of abnormal values seen after operation in both the malignant and the benign groups indicates some sort of reaction to operation. Whether this is due to tissue breakdown, blood loss, anesthesia, trauma, etc., cannot, of course, be determined.

In a few instances tests were run on blood samples taken 2–3 months post-operatively. Several of these were still abnormal. Others were normal, in one or both tests. Six of these patients had widespread metastases which could not be removed, but in five of these either one or both tests were normal 2–3 months post-operatively. This indicates that the tests would be of no value in showing whether or not a malignant growth had been successfully removed.

CONCLUSIONS

Neither the tryptophan-acid test nor the iodoacetate index is of value, singly or combined:

1. To indicate the presence of malignancy.

2. To indicate whether or not a malignant growth has been successfully removed.

The results do not indicate a connection between tissue breakdown and output of the substances giving the tryptophan-acid or the iodoacetate index tests.

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Regeneration of the Liver in Parabiotic Rats*†‡

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In the adult animal, as is well known, the various tissues of the body exhibit mitotic rates which are quite characteristic, and which seem to be geared to the needs of the organism in providing for the replacement of worn-out cells. Thus, the rate at which cells normally divide is more nearly related to their life expectancy than to their actual growth potential. The latter is, in most instances, far greater than is ever manifested under ordinary circumstances. Relatively little is known about the fundamental mechanisms which are involved in growth regulation of this kind, but they deserve careful study, since lack of such control is one of the chief attributes of cancer.

In the present investigation, an initial attempt has been made to explore the operation of these mechanisms in rat liver. In the normal adult rat the incidence of mitosis is very low; at a given instant not more than one dividing nucleus may be found among 10–20,000—a rate of 0.005–0.01 per cent (3). Following extirpation of approximately one-third of the liver, however, the rate rises to 0.05 per cent.[1] If two-thirds of the liver are removed, the rate goes up to 2 per cent or more (3)—a 200- to 400-fold increase over the normal resting value. As the number of hepatic cells approaches its original limit, the mitotic rate falls to normal. We have attempted to localize the mechanism by which this balanced response to physiological needs is maintained. An attempt has been made to ascertain whether factors circulating in the blood stream during regeneration could serve to initiate the proliferative response to partial hepatectomy, or whether the stimulus is purely local.

In order to detect the presence of blood-borne factors, a series of parabiotic rats was prepared. When the animals had reached a suitable age, and the anastomosis was well healed, one member of each pair was partially hepatectomized. After an appropriate interval, the animals were sacrificed, and the intact liver of the nonhepatectomized partner was examined for alterations known to occur characteristically during hepatic regeneration. In several instances parabiotic triplets were also prepared. In these, partial hepatectomies were carried out on the two end partners, leaving the middle one intact for subsequent examination.

METHODS

The rats used in these experiments were all derived from Wistar stock, bred in our own laboratory for the past 8 years. Males and females were used, but both partners of any one pair were of the same sex. In the first eight pairs the animals were matched as to age and weight but were not littermates; in all subsequent instances they were littermates.

All operative procedures were carried out under ether anesthesia and with the usual aseptic precautions. The fur was removed by shaving or, more frequently, by means of a depilatory (19).

Parabiotic pairs were prepared by either the technic of Bunster and Meyer (5) or by a modification of the open celomic method. By the former procedure, although the peritoneal cavities of both rats were incised, they were closed in such a manner that the abdominal walls were united without celioanastomosis. Thus, since the peritoneal cavities were kept separate, there was no opportunity for adhesions to develop directly between the viscera of the partners. When open celomic anastomosis was performed (20), the anterior and posterior flaps of the abdominal incisions were sutured in such a way that the two peritoneal cavities communicated. This latter technic was modified by an additional step; the anterior surfaces of the medial two-thirds of both spleens were lightly scarified with a scalpel and then fixed in apposition by a fine continuous silk suture run through the superior and inferior edges. This procedure was intended to increase the area of healing surfaces between the two rats and so enhance the volume of cross circulation.

Parabiotic triplets were united by the open

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celomic method, but the spleens were not joined. The entire operation was carried out in one stage.

In the earlier experiments, partial hepatectomies were performed by the usual technic, with excision of the median and left lateral lobes (8, 2). Later, to augment the regenerative stimulus by the production of an even greater liver deficiency, part of the right lateral lobe was also removed. After the main lobes had been excised in the routine manner, a heavy (No. 2) silk thread was looped about the upper third of the right lateral lobe, including the distal portions of its two component lappets, tightened, and tied securely. Although by this procedure the ligature tore its way through the middle of the lobe, very little loss of blood resulted, and the distal parts of the lobe could readily be excised.

It was originally demonstrated by Brues *et al.* (2) that the liver remaining after extirpation of the two main lobes comprised 31.6 ± 1.5 per cent of the total. Good agreement with this result has been obtained in our hands (4). Hence, the weight of the total liver was calculated on the assumption that the median and left lateral lobes constituted 68.4 per cent of its original mass. In those animals in which part of the right lateral lobe was also removed, the main lobes and the additional lobes were weighed separately, and the total original liver mass estimated as usual from the weight of the former. The total percentage excised was then determined on the basis of the combined weights of all the extirpated lobes. By this means the total amount of liver excised was found to comprise from 75 to 88 per cent of that originally present.

The animals were sacrificed under ether anesthesia by exsanguination while the liver was rapidly excised, at intervals of 24, 48, or 72 hours after completion of the partial hepatectomy.

The liver removed at the time of partial hepatectomy served as the control for the intact liver of the nonhepatectomized partner, obtained 1–3 days later at autopsy.

As soon as possible after removal of the liver (either at operation or autopsy), small pieces were fixed in Bouin's fluid for histological study, and other samples were weighed and set aside for determination of total nitrogen, pentosenucleic acid (PNA), desoxypentosenucleic acid (DNA), alkaline phosphatase, and water content.

The rats were denied food for 20–24 hours prior to partial hepatectomy or autopsy, in order to reduce the hepatic glycogen content and thus facilitate the enumeration of mitotic figures in the tissue sections, and also to provide a more uniform condition of the tissue for chemical analysis.

Following fixation, the tissues were imbedded and sectioned at 6 μ. The sections were examined at a magnification of 660× under a microscope fitted with an ocular field-stop which delineated a square 0.17 × 0.17 mm. in the object plane. For each section studied, the average number of hepatic cell nuclei per field was estimated from counts performed on ten such fields. Successive fields were searched for mitotic figures either until 30 mitoses had been found or until a total area equivalent to at least 100,000 nuclei had been examined. Only fields containing liver cells and no sizable ducts or vessels were chosen, so that the results would be uniform. Since it has been shown that the distribution of mitoses is random throughout the liver lobule (3), no error should arise from an arbitrary selection of fields. Only very obvious mitoses were counted—those in late prophase, metaphase, anaphase, or early telophase, and with a large complement of chromosomes present—to preclude enumeration of part of the same figure in an adjacent section. As an added precaution against this possibility, since nuclei undergoing mitosis may be several times as large as the thickness of the section, only every second section was studied. The total number of nuclei was estimated from the average number of nuclei per field and the total number of fields examined. The percentage of nuclei undergoing mitosis at a given instant was calculated from this value and from the total number of mitoses observed. For determination of alkaline phosphatase, the liver samples, which had been stored in glass-stoppered flasks containing 2 drops of chloroform at −10° C., were thawed, finely minced, then ground with washed sand and allowed to stand for 48 hours at 4° C. in 20 volumes of distilled water containing several drops of chloroform (7). After being strained through washed gauze, aliquots of the extract were incubated with M/200 disodium monophenyl phosphate in M/15 glycine buffer and the liberated phenol measured colorimetrically (12). Activity was expressed as milligrams of phenol liberated/gm fresh tissue/hour at pH 9.3 and 37° C.

For determination of nucleic acids, samples of liver which were not processed immediately were either frozen in small chunks by immersion in isopentane cooled in liquid nitrogen to −190° C. and then stored under isopentane at −40° C. to be homogenized later, or they were homogenized immediately in 5 volumes of distilled water and stored at −40° C. as homogenates. The procedure was a combination of the method of Schmidt and Thannhauser (21) with that of Schneider (22). The former method was followed in separating pentosenucleic acid (PNA) from desoxypentosenucleic acid (DNA), by precipitation of the DNA after

alkaline hydrolysis. The PNA, which remained in the supernatant, was determined as pentose by the orcinol reaction (16). The DNA was extracted from the precipitate by treatment with hot trichloroacetic acid according to the method of Schneider (22) and was measured as desoxypentose by the diphenylamine reaction (6). All analyses were referred to the same commercial samples of the respective nucleic acids. Measurements of optical density were made with the Beckman model DU spectrophotometer. Nitrogen analyses were performed by the micro-Kjeldahl procedure (13).

The percentage of water present was determined from a known weight of finely chopped fresh liver, which was dried to constant weight at 78° C. and a pressure of 0.05 mm. Hg. All analyses have been expressed in terms of fresh liver weight.

RESULTS

Parabiotic pairs without celioanastomosis.—The first group of parabiotic rats consisted of seven pairs of animals that were not littermates. They were united at 2–3 months of age, without celioanastomosis, by the technic of Bunster and Meyer. Seven months later approximately 68 per cent of the liver was removed from the partner on the right of each pair. Two pairs were sacrificed at 48 hours, and the rest at 72 hours after the partial hepatectomy.

At autopsy, the first three pairs of rats (Nos. 6, 7, and 8 in Table 1) were found to be loosely joined, the union involving little more than the skin, which was not well vascularized. These three pairs have been omitted from the final analysis of the results.

The autopsy findings in the remaining four pairs revealed a more effective type of union, with closer apposition of scapulas and junction of part of the abdominal musculature, except in pair No. 15. In this pair no grossly visible connection was observed between muscle layers, and a relatively low mitosis count was obtained in the liver of the nonhepatectomized partner (Table 1).

In each of these four pairs, the percentage of dividing nuclei was higher in the liver of the nonhepatectomized partner than in the control, with a mean of 3.8 times the control level, in spite of the abnormally high value found in control No. 10 for which no obvious explanation could be found.

Parabiotic pairs with celioanastomosis and splenic juncture.—This group consisted of five pairs of rats, all but the first of which were littermates. Parabiosis was produced at between $1\frac{3}{4}$ and 3 months of age and involved open celomic anastomosis and junction of spleens. After $1\frac{3}{4}$–$2\frac{3}{4}$ months, 68 per cent of the liver was removed from the right partner of each pair. Two pairs were sacrificed at 24 hours, one at 48 hours, and two at 72 hours after the hepatectomy.

At autopsy all the pairs appeared to be well joined, with skin and muscle layers firmly united. The spleens were in good apposition, and the surfaces were adherent, although the passage of blood vessels through the juncture could not be positively demonstrated microscopically.

In the two pairs of rats sacrificed 24 hours after hepatectomy, the intact livers of the nonhepatectomized partners exhibited no increase in mitotic rate as compared to the controls (Table 1). This finding was anticipated and is in keeping with the observation that mitosis is not increased in regenerating liver during the first day after partial hepatectomy (3).

TABLE 1

PER CENT OF LIVER CELLS IN MITOSIS IN CONTROLS AND NONOPERATED PARTNERS OF PARABIOTIC TWINS

Pair no.	Age at parabiosis (months)	Age at hepatectomy (months)	Hrs. from hepatectomy to sacrifice	Per cent mitosis in control	Per cent mitosis in intact partner	Ratio-partner/control
Bunster and Meyer technic; 68 per cent hepatectomy						
P6	3	10	48	0.0018	(0.0039)	
P7	3	10	72	0.0063	(0.0038)	
P8	3	10	72	0.0027	(0.0032)	
P10	3	10	48	0.0239	0.0385	1.6
P9	3	10	72	0.0042	0.0396	9.4
P14	2	9	72	0.0051	0.0143	2.8
P15	2	9	72	0.0019	0.0079	4.2
Av.				0.0066	0.0251*	3.8
Open celomic-splenic technic; 68 per cent hepatectomy						
P23	$1\frac{3}{4}$	4	24	0.0026	0.0014	
P24	$1\frac{3}{4}$	4	24	0.0049	0.0028	
P20	3	5	48	0.0049	0.0269	5.5
P25	$2\frac{1}{4}$	4	72	0.0027	0.0774	28.7
P26	$2\frac{1}{4}$	4	72	0.0021	0.0096	4.6
Av.				0.0034	0.0380†	11.2

* P6, P7, and P8 not included in this mean value (see text).
† P23 and P24 not included in this mean value (see text).

In the remaining three pairs, the intact livers of the nonhepatectomized partners all exhibited an increase in mitosis, the mean rate being 11 times that of the control animals (Table 1).

Parabiotic pairs with celioanastomosis and splenic juncture given colchicine.—In the third group of parabiotic pairs, an unsuccessful attempt was made to augment the effect obtained in the first two groups. A larger fraction of the liver was excised in the hope of producing a stronger stimulus, and colchicine was administered 8–12 hours before sacrifice in order to produce an accumulation of mitoses and to facilitate counting.

There were five pairs of rats in this group, all littermates, united at between 3 weeks and 1 month of age by celioanastomosis and splenic juncture. Partial hepatectomies were performed 2, 4, or 12 months later, and instead of the usual 68 per cent, approximately 80 per cent of the liver was excised from the right partner of each pair.

The use of colchicine in this experiment necessitated the use of separate controls, since it was undesirable to administer the drug twice to the same pair of rats. The controls in this instance were intact separate rats of the same age and sex as the corresponding parabiotic pair, injected with colchicine and subsequently sacrificed at the same time. Three pairs were sacrificed at 48 hours and two pairs at 72 hours after the hepatectomy. The dose of colchicine was 0.02 mg. in the first three and 0.10 mg. in the latter two pairs, as noted in Table 2; both rats in each pair were injected with these amounts of the drug.

In the two pairs receiving the high dosage, both of the hepatectomized partners were found dead at the time the nonhepatectomized partners were sacrificed; however, this dosage appeared to be well tolerated by the latter and by the controls. The manifest toxicity of the drug in liver-deficient animals may have seriously limited the size of the mitotic stimulus transmitted to the intact partner. Nevertheless, in all five instances in this group, mitoses in the livers of the nonhepatectomized partners were more numerous than in their corresponding controls, the average value for the former exceeding the latter by more than fourfold (Table 2). Thus, while no augmentation of the previous effect was achieved, these findings confirm the positive results obtained in the first two groups.

The results of these experiments are shown graphically in Chart 1.

Parabiotic triplets with celioanastomosis.—Another means of increasing the relative amount of liver deficiency was afforded by the use of parabiotic triplets, in which partial hepatectomies could be performed on two rats out of three.

Triplets were prepared from littermates united by celioanastomosis at 1–1½ months of age. Three sets survived in good condition and were subjected to partial hepatectomy at 2, 4, and 6 months of age, respectively. From 75 to 80 per cent of the liver was removed from the two end partners of each set, leaving the middle one intact. All three sets were sacrificed 48 hours after the partial hepatectomies.

The results were striking (Table 3). The percentage of nuclei in mitosis was from 44 to 62

TABLE 2

PER CENT OF LIVER CELLS IN MITOSIS IN CONTROLS AND NONHEPATECTOMIZED PARTNERS OF PARABIOTIC TWINS AFTER COLCHICINE

(Open celomic-splenic technic; 80 per cent hepatectomy)

Pair or control no.	Age at parabiosis (months)	Age at hepatectomy (months)	Hrs. from hepatectomy to sacrifice	Per cent of liver excised	mg. of colchicine	Hrs. from colchicine to sacrifice	Per cent mitosis in control	Per cent mitosis in intact partner	Ratio partner/control
P32	1	5	48	79.3	0.02	12		0.0175	4.3
PC7		5		0	0.02	12	0.0041		
P33	¾	5	48	84.4	0.02	12		0.0302	4.0
PC8		5		0	0.02	12	0.0076		
P29	1	13	48	83.9	0.02	8		0.0526	39.1
PC13		13		0	0.02	8	0.0016		
P30	1	3	72	87.5	0.10	11		0.0152	1.1
PC2		3		0	0.10	11	0.0133		
P31	1	3	72	84.5	0.10	11		0.0179	4.0
PC3		3		0	0.10	11	0.0045		
AV.							0.0062	0.0267	4.3

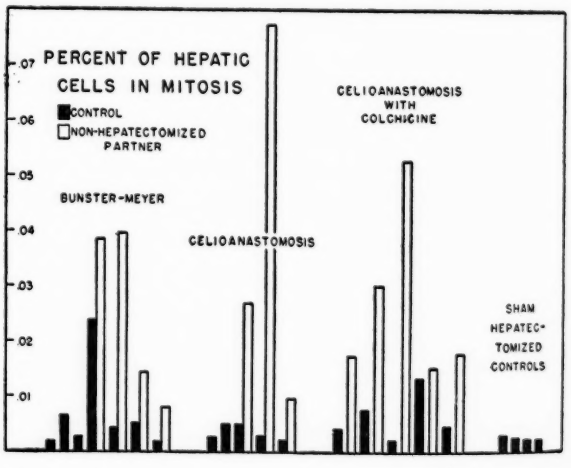

CHART 1.—Percentage of hepatic cells in mitosis in controls and nonhepatectomized partners of parabiotic twins grouped as in Tables 1, 2, and 4.

times the mean control value for each individual set, or a mean increase of 50-fold. The results of all the above experiments are summarized graphically in Chart 2.

Sham-hepatectomized and parabiotic controls.—Four normal, separate female rats, 4 months of age, were subjected to sham operations which simulated a partial hepatectomy; laparotomies were performed, followed by manipulation of the liver, and excision of a very small biopsy. The animals were sacrificed 48 hours later.

The mean mitotic rate in the livers of these rats was found to be 0.0025 per cent (Table 4), as compared to 0.0057 per cent for the controls in all the parabiotic series except those which received colchicine. Thus, the increase in mitosis found in the nonhepatectomized partners in the above experiments could not be attributed to factors attendant upon the operative procedure *per se*. The difference between the mean value for these normal, separate controls and that for the parabiotic controls is probably unimportant, since 0.0057 per cent is within the range reported elsewhere for normal adult rats (3).

Although the basic mitotic rate was not altered by the state of parabiosis, approximately 20 per cent of the livers in these animals exhibited underdevelopment or atrophy of the caudate lobes. This anomaly appeared sometimes in either partner, and sometimes in both. Whatever its cause, since long intervals of time elapsed between the parabiotic union and the final stage of the experiment, no interference with the eventual outcome seemed likely.

Results of biochemical studies.—The results of the biochemical studies are shown in Table 5 and will be summarized very briefly, since the changes produced in the intact liver of the nonhepatectomized partner in these experiments were for the most part too slight to be reflected by gross alterations in chemical composition. The water content, total nitrogen, alkaline phosphatase, and DNA all remained within normal limits in the livers of the nonhepatectomized partners. The PNA showed a suggestive but not highly significant rise in the experiments with parabiotic twins ($0.02 < P < 0.05$), but not with the triplets. In the lat-

TABLE 3

PER CENT OF LIVER CELLS IN MITOSIS IN CONTROLS AND NONHEPATECTOMIZED PARTNERS OF PARABIOTIC TRIPLETS

(Open celomic technic; 80 per cent hepatectomy on right and left partners)

Triplets no.	Partner	Age at parabiosis (months)	Age at hepatectomy (months)	Hrs. from hepatectomy to sacrifice	Per cent of liver excised	Per cent mitosis in controls	Per cent mitosis in intact partner	Ratio partner/controls
P37	Right	1½	4	48	79.2	0.0069		
	Left	1½	4	48	80.1	0.0163		44
	Middle	1½	4	48	0		0.5128	
P41	Right	1	6	48	77.8	0.0036		
	Left	1	6	48	77.5	0.0085		58
	Middle	1	6	48	0		0.3546	
P53	Right	1¼	3	48	79.1	0.0038		
	Left	1¼	3	48	75.1	0.0009		62
	Middle	1¼	3	48	0		0.1475	
AV.						0.0067	0.3383	50

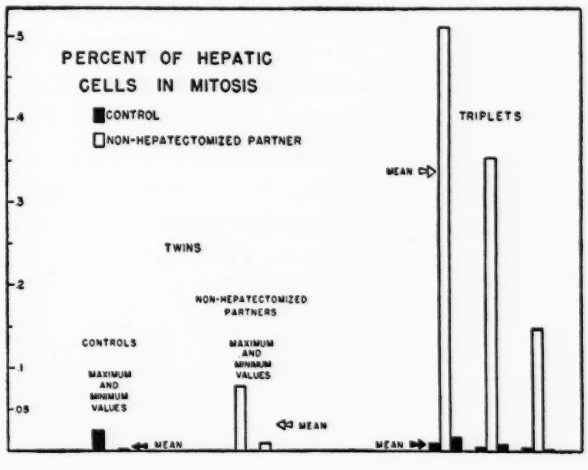

CHART 2.—Percentage of hepatic cells in mitosis in controls and nonhepatectomized partners of parabiotic triplets. Note that the scale is reduced by a factor of 15 in comparison with Chart 1. The maximum, minimum, and mean values for Chart 1 are included, to clarify this relationship.

TABLE 4

PER CENT OF LIVER CELLS IN MITOSIS IN SHAM-HEPATECTOMIZED CONTROLS

Rat	Age (months)	Hrs. from laparotomy to sacrifice	Per cent mitosis
PC9	4	48	0.0029
PC10	4	48	0.0025
PC11	4	48	0.0022
PC12	4	48	0.0022
Av.			0.0025

TABLE 5

Per Cent Restoration of Liver Mass, and DNA, PNA, Alkaline Phosphatase, Total Nitrogen and Water Content of Control, Regenerating, and Intact Livers of Parabiotic Twins and Triplets

Rats no.	Hrs. from hepatectomy to sacrifice	Per cent restoration* of liver mass	Desoxypentosenucleic acid†‡			Pentosenucleic acid†§			Alkaline phosphatase‖			Total nitrogen per cent fresh wt.#			Water content, per cent**		
			Control	Regenerating	Intact	Control	Regenerating	Intact	Control	Regenerating	Intact	Control	Regenerating	Intact	Control	Regenerating	Intact
Twins: Bunster and Meyer or Open Celomic-Splenic Technic; 68 Per Cent Hepatectomy																	
P23	24	43.2		150	182	784	1,021	952	1.36	6.88	1.44	3.46	2.93	3.27	70.3	68.0	70.7
P24	24	44.7	155	121	166	1,068	1,055	795	1.41	7.80	1.80	3.44	3.33	3.52	69.8	68.4	70.0
Mean††		43.9		136± 14.6	174± 8.1		1,038± 17.1	874± 79.3		7.34± .465	1.62± .181		3.13± .202	3.39± .126		68.2± .200	70.4± .360
P6	48		172			609			2.50			3.83			67.6		
P10	48	66.5	128	124	238	922	1,151	830	2.10	4.60	1.60	3.74	3.62	3.39	68.3	66.6	68.6
P20	48	55.0	264	284	259	744	1,075	1,045	1.90	2.48	1.34	3.48	2.86	3.57	70.2	71.4	70.2
Mean††		60.7		204± 80.7	248± 10.6		1,113± 38.4	938± 108.6		3.54± 1.070	1.47± .131		3.24± .382	3.48± .091		69.0± 2.38	69.4± .810
P7	72		196			793			0.70			3.26			71.8		
P8	72		196			861			0.45			3.20			72.0		
P9	72	68.2	169	126	221	908	1,031	955				3.41	3.11	3.44	70.8	72.8	69.8
P14	72	68.7		116	224	700	655	935				3.64	2.87	3.39	69.5	72.3	69.8
P15	72	66.0	239	104	203	785	1,018	987				3.43	3.33	3.59	70.6	72.8	69.4
P25	72	61.2	106	170		631	987					3.40	2.82	3.36	71.2	72.6	70.7
P26	72	53.0	123	192	236	618	978	845	0.64	1.60	0.96	3.45	3.12	3.45	71.6	70.3	69.4
Mean††		63.4	175± 15.9	142± 17.1	221± 6.8	785± 40.0	934± 71.5	931± 30.5	1.38± .270			3.48± .052	3.05± .094	3.45± .040	70.3± .390	72.2± .481	69.8± .187
Triplets: Open Celomic Technic; 80 Per Cent Hepatectomy																	
P37R	48	24.8						1,075	0.64	6.43	0.73					71	78
L	48	31.2				1,050			0.59	4.37						70	
P41R	48	39.0				1,000		1,055							71.4		66.4
L	48	26.0				1,020									70.7		
P53R	48	40.8				990		1,022							72.5		68.2
L	48	44.0				990									72.8		
Mean††		34.3				1,010± 18.6		1,051± 223.0	0.62± .025	5.40± 1.040						71 ± .714	70.8± 2.54

* Calculated according to the method of Brues, Drury, and Brues (2).

† Mg/100 gm of fresh tissue.

‡ Standard error of the method = ± 7.5 per cent.

§ Standard error of the method = ± 3.9 per cent.

‖ In units = mg of phenol liberated/gm of fresh tissue/hr at pH 9.3 and 37° C.

Standard error of the method = ± 2.1 per cent.

** Standard error of the method = ± 0.9 per cent.

†† Mean ± standard error of the mean.

ter, the state of parabiosis appears to be attended by more sweeping alterations in nutritional status and other physiological functions than in the former. Hence, in triplets the pattern of the biochemical responses of the liver to the regenerative stimulus may not necessarily be the same as in the twins.

It should be noted that the mitotic rate, which can increase by 400-fold during hepatic regeneration, is at least 50 times more sensitive as an indicator of regenerative activity than any of the biochemical tests employed.

Results obtained in the regenerating livers of partially hepatectomized partners.—The effect of parabiosis on the regenerative process in the partially hepatectomized partners could best be evaluated in the animals that were subjected to 68 per cent hepatectomies, since the response in separate rats has been widely studied under these circumstances and is predictable. Restoration of liver mass (Table 5) was found to proceed at the same rate as that obtained in previous experiments in this laboratory in which normal separate rats were studied (4). Mitotic figures were not counted, but they were present in sufficient numbers to indicate that active proliferation was in progress. The rate of restoration of hepatic nuclei (2, 4) determined in one liver that had been regenerating for 72 hours (Rat 25, Table 1) was found to be at the expected level for separate rats (43 per cent). The biochemical alterations exhibited by these regenerating livers (Table 5) were likewise consistent with those reported to occur in hepatic regeneration in normal separate rats (2, 17, 18).

In the animals subjected to 80 per cent hepatectomies, the progress of restoration was more difficult to evaluate, because of lack of a suitable reference standard. The regenerating remnant increased in size and became excessively fatty, assuming a pale cream color. On microscopic examination, mitotic figures were very numerous, but whether the triplets differed significantly from the twins in this respect could not be determined because of the variability in the amount of liver removed by this technic.

In general, the results implied that the proliferative response in the livers of the intact partners was not of sufficient magnitude to inhibit appreciably the regenerative response in the hepatectomized partners.

DISCUSSION

The above experiments have demonstrated that excision of part of the liver from one parabiotic partner was followed in every instance by a proliferative response, not only in the liver of the partially dehepatized rat but also, though to a lesser degree, in the intact liver of the other partner. These findings provide evidence in favor of the existence of a blood-borne cog in the mechanism for the initiation of liver mitosis.

Although the results were consistently positive, the nonhepatectomized partners in all groups exhibited considerable variation in the degree of this mitotic response. Similar variability has been noted in the regenerating livers of normal separate rats in which it was observed that the percentage of cells undergoing mitosis fluctuated widely from hour to hour and at different times in different individuals (3). Differences in the efficiency of the cross-circulation between partners may have also contributed to the variability of our results. Even when the same technic of anastomosis has been employed, the rate of blood exchange between partners varies somewhat in different pairs of rats (1, 25). For an agent to serve effectively in carrying a stimulus from one partner to another via the blood stream, the rate of transmission from donor to recipient must exceed the rate of elimination in the recipient partner by an amount sufficient for an above-threshold concentration of the active substance to obtain in the latter (11). In our experiments with parabiotic twins, where the stimulus was not very great, small differences in the volume of blood exchanged may have exerted an appreciable effect upon the degree of response exhibited by the nonhepatectomized (i.e., recipient) partner.

With parabiotic pairs, the results, although definite and clear-cut, were not striking. The mean mitotic rate for the livers of nonhepatectomized partners in all groups of twins (0.03 per cent) exceeded that of the controls (0.005 per cent) by approximately sixfold. If 68–80 per cent of the liver is removed from one rat of a parabiotic pair, the total liver mass removed from both is only 34–40 per cent. Thus, the rise in mitotic rate to 0.03 per cent in the nonhepatectomized twins is compatible with the finding of Hempelmann, previously mentioned,[1] that when only one-third of the liver was removed from a single rat, the mitosis rate approached 0.05 per cent—as opposed to 2 per cent when two-thirds were removed.

A considerable augmentation of the positive effect obtained in the experiments with parabiotic twins occurred with triplets. In the latter, since 80 per cent hepatectomies were performed on two rats out of three, the total liver mass removed from the trio was approximately 53 per cent—an appreciable increase over the 34–40 per cent

[1] L. H. Hempelmann, personal communication.

removed from the twins. In addition to the larger stimulus provided by the greater liver deficiency, a more effective cross-circulation should occur in triplets, since the anastomosis in the middle partner is twice as extensive as in twins. It appears from these data, as well as from those of Hempelmann cited above, that a large stimulus is necessary in order to obtain a substantial response.

To insure against unpredictable alterations in the mitotic rate that might arise as an accompaniment of the parabiotic state, one partner of each pair was used as the control for the other. Thus, any effect resulting from parabiosis itself would be reflected in the control. The atrophy of the caudate lobes, exhibited by many rats in our series, had no detectable effects upon the mitotic rate, since the control rats in which it occurred exhibited mitotic counts well within normal limits. Hence, the presence of this anomaly could not be accountable for any significant experimental error.

In the present experiments, the parabiotic technic has been employed to explore the role of the blood stream in transmitting the mitotic stimulus which initiates hepatic regeneration under physiological conditions. Significant studies on the relation of circulation to hepatic regeneration have been previously reported by Mann and his co-workers. In an ingenious series of experiments involving Eck fistulas, partial and reverse Eck fistulas, and partial ligation of the portal vein, they demonstrated that the amount of regeneration was related directly to the amount of portal blood flow and did not depend upon the physiological needs of the organism (14, 9, 24, 10, 15). The marked distention of the sinusoids in the liver remnant, resulting from the necessity of accommodating the normal volume of portal blood, was believed to induce a coincident hypertrophy of the hepatic cells, with an accompanying increase in mitosis. A second possible interpretation of these findings, however, is that rather than purely mechanical distention of the sinusoids by an added flow of portal blood, there are chemical entities in the blood which, when present in sufficient amounts, may cause a proliferative response in the liver. Thus, it is conceivable that either an elevated concentration of such substances or an increased flow of blood bearing the same concentration would be equally effective. Our results tend to support this second interpretation, since we do not believe that the volume of blood circulating through the intact liver of the nonhepatectomized partner has been altered in our experiments. In only one instance was there any evidence of an adhesion, and that was between the liver and spleen of one of the rats in a colchicine-treated pair. In all other cases, and particularly in the animals joined by the Bunster and Meyer technic without celioanastomosis, no adhesions were detected between the organs drained by the portal system and the body wall. Such adhesions would have to be quite extensive, and present in both partners, to produce an effective increase in blood flow through the liver of one partner as a result of a hepatectomy in the other.

SUMMARY AND CONCLUSIONS

Partial hepatectomies have been performed on one partner of each of fourteen parabiotic pairs, and on two partners of each of three sets of parabiotic triplets.

At intervals of 48 or 72 hours later, the intact livers of the nonhepatectomized partners all exhibited a higher mitosis rate than the control livers removed at the time of hepatectomy. In parabiotic twins the mean value was 6 times, and in triplets it was 50 times, that of the controls.

Two of the fourteen pairs were sacrificed at 24 hours, and, in these, no significant alteration occurred in the livers of the nonhepatectomized partners.

Biochemical determinations of alkaline phosphatase, total nitrogen, DNA, and water content failed to demonstrate significant differences. PNA values were increased slightly above the control levels in the nonhepatectomized partners of the twins but not in the triplets. All these entities are far less sensitive indices of proliferative activity than the mitotic count.

The evidence suggests that in regenerating rat liver, mitosis is initiated by alterations in the chemical composition of the blood.

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The Transformation of a Solid Transplantable Mouse Carcinoma into an "Ascites Tumor"

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The Ehrlich ascites tumor of mice has been demonstrated to be an exceptionally valuable tool for quantitative investigations on the growth and biochemistry of neoplastic cells (3). On the basis of the many advantages which, for certain investigations, the ascites tumor presents over solid tumors, it was believed that it would be of great value if tumors other than the Ehrlich carcinoma could also be grown as ascites tumors. In the present work a solid transplantable mouse carcinoma was studied with respect to its intraperitoneal growth. After a number of transfers of the exudate, a typical ascites tumor was obtained, and information was gained as to the possible mechanism of this transformation from the solid growth to the growth as a cell suspension.

The term "ascites tumor" must first be defined: It refers to neoplasms growing in the form of suspensions of free and homogeneously distributed living tumor cells in the ascitic fluid, in cases where the proportion of tumor cells (after a certain stage of development) is very high, representing nearly a pure culture. It is understood that there is always some infiltration of solid tissues in the peritoneal cavity as well. The amount of solid infiltration varies inversely with the number of tumor cells inoculated and depends upon the time of survival, which in turn is dependent on the rate of ascites tumor development. This infiltration is microscopic in certain cases, macroscopic in others, and results sometimes in huge, solid tumors. Under certain well defined conditions the inoculation of certain numbers of cells will produce mostly or only solid tumors of the peritoneal cavity. These, of course, will not be called ascites tumors.

There are several reports in the literature of different tumors, besides the Ehrlich carcinoma, which have been grown as more or less pure cultures of tumor cells in the peritoneal fluid. (For references about the Ehrlich ascites tumor see [3].) Thus, Koch was able to grow the Flexner-Jobling rat carcinoma in the peritoneal cavity as an ascites tumor, with variable amounts of solid tumor formation (6). Krebs *et al.* have obtained a typical ascites tumor after the intraperitoneal inoculation of a lymphosarcoma in mice (7). The peritoneal fluid contained the lymphoblast-like tumor cells in practically pure culture; there was also a solid infiltration of the fatty tissue in the peritoneal cavity. The tumor was readily transmitted by the ascitic fluid. Yoshida has described a rat sarcoma, originating in the scrotum, which spontaneously infiltrated the peritoneal cavity and gave rise to a typical ascites tumor (11). Extensive work has been done on this tumor by different Japanese workers. Yoshida and Sazaki (11) tried to produce artificially other ascites tumors in mice and rats by intraperitoneal inoculation of solid tumor emulsions, but they consistently failed to get other than solid growth. Very recently, Goldie and Felix (2) succeeded in producing growth of free tumor cells in the peritoneal fluid with Sarcoma 37 and a spontaneous thymoma in a DBA mouse. They studied the growth characteristics of these cells in detail.

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PROCEDURE AND RESULTS

By the courtesy of Prof. C. Krebs (Aarhus) we obtained the Krebs-2 solid carcinoma, which arose spontaneously in the inguinal region of a hybrid male mouse and has been kept since then by subcutaneous transfers in hybrid white mice (10). After a number of transplant generations, the tumor was found (8) to take in 100 per cent of the animals and showed no spontaneous regressions.

We inoculated 0.2–0.4-ml. amounts of tumor pulp from 12-day-old tumors intraperitoneally into nine white mice (Chart 1). Solid tumors of the peritoneal cavity and/or the abdominal wall developed in all inoculated animals, and they died in 21–42 days. In one out of nine animals, however, besides a huge solid tumor, the formation of a hemorrhagic exudate was noted 36 days after the inoculation. This exudate, which amounted to about 1 ml., contained mainly inflammatory cells. The cells of the fluid were, as in all subsequent cases, differentiated on smears prepared according to the method of Papanicolaou. Many polymor-

phonuclear leukocytes, lymphocytes, histiocytes, and some mesothelial cells were present. No tumor cells could be detected in the fields examined (Fig. 1).

This hemorrhagic fluid was injected in 0.3-ml. amounts into two animals. Both animals developed solid tumors, which, again, were accompanied by the formation of an exudate very similar to that obtained in the first transplant generation. These exudates were collected 28 days after inoculation and were injected into four animals in 0.5-ml. amounts. All animals in this group developed solid tumors plus a peritoneal exudate. One of these exudates, which was collected 31 days after inoculation, differed conspicuously, however, from the others (which were similar to the previous ones), in that it contained well distinguishable and huge tumor cells with abundant chromatin and large nucleoli. These cells represented 10 per cent of all the cells present (Fig. 2). Amounts of this fluid (0.5 ml.) were then intraperitoneally inoculated into five animals. Of this group, one mouse died of a solid tumor without exudate formation after 24 days. Two animals developed solid tumors accompanied by inflammatory exudates and died after 14 and 16 days, respectively. The remaining two animals with exudates which contained about 50 per cent tumor cells (Fig. 3) showed a widespread infiltration of the peritoneal cavity, but no huge solid tumor was present. These animals died as early as 10 days after inoculation. The transfer of the fluids from these animals, finally, gave rise to a typical ascites tumor with 80–90 per cent tumor cells in all 6 animals injected (Figs. 4, 5, 6). This ascites tumor has been maintained for the past 5 months without change in its character. Subcutaneous injection of the fluid produced solid tumors, similar in structure to the original Krebs-2 tumor. The Krebs-2 ascites tumor has been compared to the Ehrlich ascites tumor. The two tumors are very similar with regard to all characteristics studied (Table 1), with the exception of the growth in Swiss mice. While the Ehrlich tumor showed a rather poor growth in Swiss mice with prolonged survival time, increased frequency of inflammatory cells and of solid tumor formation, and in some cases even spontaneous regressions, no change was observed in the growth of the Krebs-2 ascites tumor when injected into Swiss mice. In the A strain of mice both tumors grew equally well. It is of special interest that the desoxypentosenucleic acid (DNA) value per cell is higher in the newly established Krebs-2 ascites tumor than in normal somatic mouse cells (9), as was also established for the Ehrlich carcinoma (1). We have pointed out previously that various tumors may behave differently in this respect (4).

To investigate the relationship between solid infiltrative growth on the peritoneum and growth of the tumor cells in the ascitic fluid, a separate study was performed. Primarily, this study was

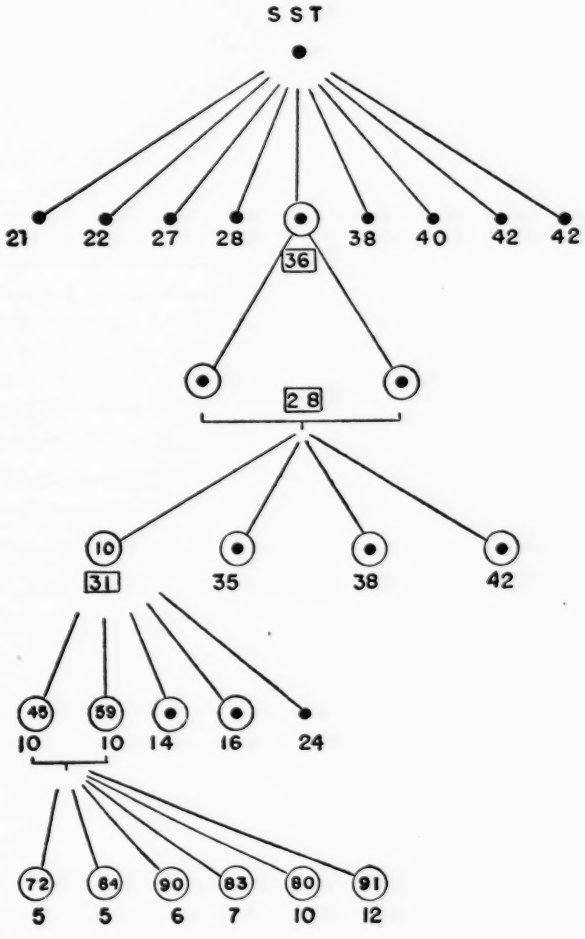

CHART 1.—Development of the Krebs 2 ascites tumor. S.S.T.: intraperitoneal injection of pulp from solid, subcutaneous tumors.

●: only solid tumor development.

⊙: exudate formation with no detectable tumor cells.

The numbers within the circles indicate the per cent of tumor cells in the exudate (see text).

The number below the circles denotes survival time in days. If within a square, it denotes the day when the animal was killed and the exudate collected.

carried out on the Ehrlich carcinoma, as the growth of that particular tumor has been investigated previously, and the survival curves of groups inoculated with different numbers of cells have been established (1, 4). In addition to the previously published findings which have shown that the survival time of the animals depends upon the number of cells inoculated, there was a definite correlation between the amount of solid infiltration in the peritoneal cavity and the sur-

vival time. Larger numbers of cells which produced 100 per cent mortality in 8–10 days (about 20×10^6 cells) gave rise to ascites tumors with no gross and very little microscopic infiltration of the peritoneum and subperitoneal fat. With decreasing numbers of cells and, consequently, increasing survival times (about 14–20 days survival, produced by $2.0–0.8 \times 10^6$ cells), a considerable gross infiltration was present in the mesenterium, omentum, peritoneal fat, etc. In all these cases a typical ascites tumor was also present. With still smaller numbers of cells and survival times over 20 days, huge solid tumors were formed in the peritoneal cavity; ascites formation was present in some cases, absent in others. Where ascites formation occurred, increased frequency of nontumorous exudate cells was often noted.

The observations described in the preceding paragraph for the Ehrlich ascites tumor were essentially the same for the Krebs-2 ascites tumor.

TABLE 1

COMPARISON BETWEEN THE EHRLICH AND THE KREBS-2 ASCITES TUMORS

	Ehrlich	Krebs 2
Average cell number/ml	134×10^6	142×10^6
Frequency of nontumorous cells*	10 per cent	17 per cent
Median survival time in days of animals inoculated with 0.8×10^6 cells	11	9
Pentosenucleic acid/cell (for methods see [1])	$2.48–2.68 \times 10^{-6}$ μg. P	$2.16–2.60 \times 10^{-6}$ μg. P
Desoxypentosenucleic acid/cell (for methods see [1])	$1.32–1.42 \times 10^{-6}$ μg. P	$1.14–1.37 \times 10^{-6}$ μg. P
Frequency of tumor cells in mitosis	3 per cent	2.5 per cent
Growth in the A strain of mice	good	good
Growth in Swiss mice	poor	good

*Counted around median survival time. Mean of 20 samples from A mice.

It seems probable that large numbers of virulent tumor cells multiply freely in the peritoneal fluid, and their overwhelming effect kills the animals within a short time. This short survival time prevents the formation of more voluminous solid tumors. With smaller numbers of cells the effect is much more gradual, with solid growths developing and the animal building up its response, so that the inflammatory reaction is much more pronounced. With inoculations of very small numbers of cells, the tumor cells in the fluid seem to be destroyed, since no ascites but only solid tumors are produced in most cases.

Ascites tumors can thus be produced by other

DISCUSSION

When first injected, the solid tumor pulp gave rise to solid tumor formation which, in one case, was accompanied by an inflammatory exudate. No tumor cells could be detected in the exudate, but some must have been present, because upon further transplantation of the fluid the tumor was transmitted. In the following transfers, the tumor cells became evident and showed a gradual increase over other cell elements during the subsequent transplant generations. It is supposed that an artificial selection of the particular tumor cells which were able to live in a fluid medium occurred during the transfer of the exudates. Since the frequency of such tumor cells increased, a larger number of cells was inoculated during each subsequent transfer; instead of several solid tumors, accompanied by an inflammatory exudate, a typical ascites tumor with only minute infiltrations was developed. This course of events could be reversed, i.e., solid tumor growth with mainly inflammatory exudates produced, (a) if the cells of the once established ascites tumor were injected in smaller numbers; or (b) if they were injected in the usual large numbers but the virulence of the cells reduced by artificial means such as storage; or (c), if the cells were injected into more resistent hosts, as in the case of the Ehrlich tumor into Swiss mice.

than the previously known tumors. Their production seems to be dependent upon the ability of the tumor cells to survive and proliferate in the fluid medium of the peritoneal exudate and, also, upon the number of such tumor cells present in the inoculum. If ascites tumors are to be used for biochemical studies, or studies on influences upon tumor growth, the number of cells in the inoculum must be sufficiently high to produce a uniformly short survival time and an ascites which contains a nearly pure culture of tumor cells. With lower numbers of cells, the interfering factors of the solid growth, the immunological response of the animals, and the longer survival time will produce a very high variability.

SUMMARY

Because the Ehrlich ascites tumor possesses certain advantages for some quantitative studies on the growth rate and the chemical components of tumor cells (3), the mechanism of the transformation of another solid transplantable mouse carcinoma into an ascites tumor was investigated.

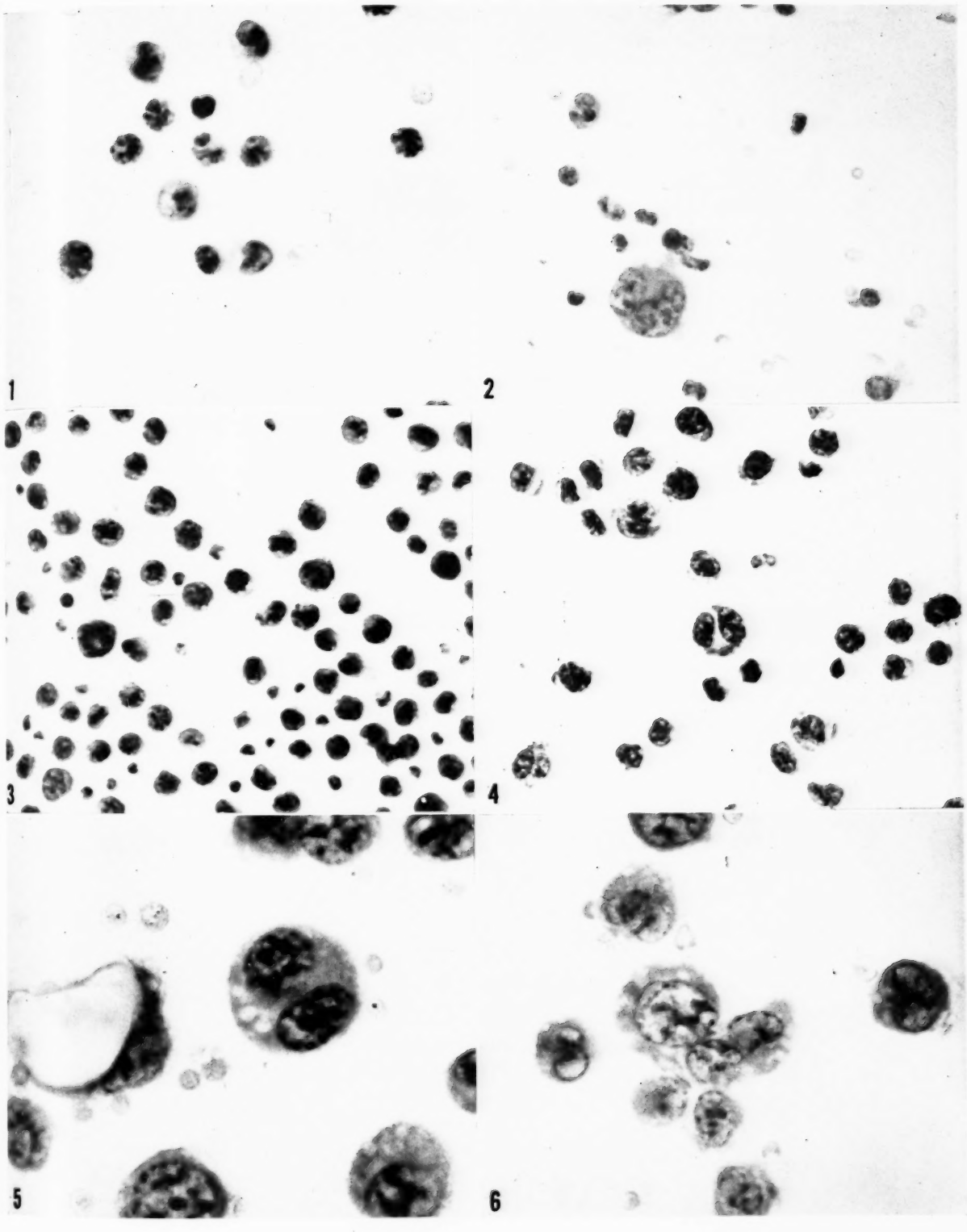
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FIG. 1.—Inflammatory exudate. Papanicolaou's smear (as in all following Figs.). Mag. ×1,100.

FIG. 2.—Field from exudate containing 10 per cent tumor cells. Note single binucleated tumor cell among inflammatory cells. Mag. ×600.

FIG. 3.—Field of exudate containing 50 per cent tumor cells on an average. Mag. ×340.

FIGS. 4, 5, 6.—Smears from the final Krebs-2 ascites tumor. Mag. ×340 (Fig. 4); ×1,100 (Figs. 5 and 6).

After the intraperitoneal inoculation of solid tumor masses, a peritoneal carcinomatosis developed. The peritoneal exudates produced were used for subsequent intraperitoneal injections through several transplant generations. A gradual increase in the relative frequency of tumor cells in the fluid could be observed, and, finally, the nearly "pure culture" state of tumor cells could be reached—which corresponds to the definition of "ascites tumor." Several characteristics of this newly produced ascites tumor are compared to the classical Ehrlich ascites tumor. The relationship between solid growth in the peritoneal cavity and growth of the cell suspension in the ascitic fluid are shown to be dependent upon the initial number of cells inoculated. The mechanism of the transformation into an ascites tumor is discussed.

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The Incorporation of Radioactive Orotic Acid into the Nucleic Acid Pyrimidines of Animal and Human Tumors*

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The incorporation of radioactive orotic acid *in vitro* into the pyrimidines of the nucleic acid of animal tissues has been studied (6, 7). In this paper data are presented on the incorporation of radioactivity into the pyrimidines of the nucleic acids of animal and human tumors. It is impossible to make any more than tentative conclusions on the differences of tumor tissue from the "normal tissues" for a variety of reasons: the variation from one normal tissue to another, the lack of homogeneity of the specimens studied, and the difficulties in adhering to any rigid microscopic criteria. On the other hand, in this system, as in many others, a certain constancy in the pattern of chemical reaction has been noted regardless of the tissue of origin of the tumor.

METHODS

Among the animal tissues studied were a rat Walker carcinoma 256, a liver from a rat bearing a subcutaneous Walker carcinoma, and a regenerating liver from a normal rat. The human tumors examined were a rapidly growing fibrosarcoma, an adenocarcinoma of the large bowel, a gastric adenocarcinoma, and a teratoma of the testis. Immediately following surgical removal, 6–10 gm. of the tumor were placed on ice, and the following procedure carried out quickly.

Two to 3 gm. of tissue were sliced with a Stadie slicer and suspended in 20 ml. of Krebs saline with added phosphate buffer at pH 7.4; from 1 to 3 mg. of radioactive orotic acid (labeled in the 2-position with C^{14}) with a specific activity of 26,000 counts/min/mg were placed in the medium and incubated at 38° C. for 4 hours in the presence of a 95 per cent O_2–5 per cent CO_2 mixture. After incubation, the slices were washed with water by centrifugation several times, homogenized, and the proteins precipitated with an equal volume of 10 per cent trichloroacetic acid. The residue was heated with 30-ml. portions of alcohol and ether until the supernatant following centrifugation was clear and colorless.

The residue from this procedure was placed in a large test tube, 10 ml. of 10 per cent aqueous sodium chloride solution was added, and the mixture was heated on a water bath for 24 hours with constant stirring. The solution was filtered and the nucleic acid precipitated by adding 2.5 volumes of absolute alcohol. After the solution had been in the cold room for 24 hours, the precipitate was removed by centrifugation, washed with absolute alcohol and ether, and dried in the desiccator. The nucleic acid was added to 0.5 ml. of 1 N HCl and heated for 1 hour in a boiling water bath. This resulted in a solution containing the free purine bases and the intact pyrimidine nucleotides.

Equal amounts of the hydrolysate were placed in a narrow band near the base of each of four strips of Whatman No. 1 filter paper, 12×40 cm., and allowed to dry. The papers were set up as ascending columns, with a 70 per cent solution of tertiary butyl alcohol (in water) made 0.8 N with HCl and run for 48 hours (5). The bands formed were easily outlined with a pencil when exposed to a "mineralight" lamp that emits most of its light in the region of 250–270 mμ. The bands in order from the solvent front down were thymidylic acid, uridylic acid, cytidylic acid, adenine, and guanine. Each band was cut out and eluted with distilled water. The solution was made slightly alkaline and placed on a column of Dowex 1, 300–400 mesh, an anion exchange resin, as employed by Cohn (2). The column was washed thoroughly with water. Ribose cytidylic acid may be eluted from the column with 0.002 N HCl,

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uridylic acid with 0.01 N. Thymidylic acid appears in at least two fractions. One portion is eluted from the thymidylic acid band with 0.01 N HCl, the other with 0.1 N HCl. In spleen and regenerating liver it is apparent that they show different specific activities. They gave the same absorption maxima. We assume at present that they represent two types of thymidylic acid. In the other tissues thymidylic acid eluted with 0.1 N HCl was analyzed and reported. Desoxyribose cytidylic acid is eluted from the column with 0.01 N HCl. The previous paper separation prevents contamination with uridylic acid. Further analysis of these methods will be presented elsewhere. Five- or 10-ml. portions of the eluates were examined for purity and quantity in the Beckman spectrophotometer. Small amounts of material not yet identified have also been obtained.

The reasons for the use of a separation based on a combination of paper and resin have been reported previously (7). It has been shown that contamination with radioactive orotic acid does not occur and that radioactivity occurs only in the pyrimidines and not in the sugar or the purines (7).

Eluates were evaporated to dryness and counted in a windowless counter. Sufficient counts were made to reduce the statistical error of counting to less than 5 per cent, except in several low counts on thymidylic acid in which the possible error is 20 per cent.

Orotic acid labeled in the 2-position was synthesized according to the method of Nyc and Mitchell (3) from cyanate made from C^{14}-NaCN. A-methopterin was kindly provided by the Lederle Laboratories.

RESULTS

Table I shows the results of incubation of slices of two normal rat livers and a normal cat liver. It will be noted that the uridylic acid had 2–4 times the specific activity of the cytidylic acid. This difference has been consistent in many similar experiments involving brain, kidney, and liver from several different species. In several cases of spleen the uridylic acid was 6–7 times as active as the cytidylic acid, but in all other cases of normal tissue it has been a two- to three-fold difference. All tissues studied in this laboratory have shown degrees of incorporation of orotic acid into the nucleic acid equal to or greater than the degrees of incorporation reported in this paper.

TABLE 1

SPECIFIC ACTIVITIES OF PYRIMIDINE NUCLEOTIDES OF NUCLEIC ACID FROM TISSUE SLICES INCUBATED WITH RADIOACTIVE OROTIC ACID

Tissue	Thymidylic acid		Uridylic acid		RNA cytidylic acid	
	Amt. (μg.)	ct/min/mg free base	Amt. (μg.)	ct/min/mg free base	Amt. (μg.)	ct/min/mg free base
Rat liver (1)	120	21	1,835	358	3,006	85
(2)			1,460	410	2,280	207
Cat liver	103	45	1,088	795	1,946	224
Rat Walker carcinoma 256	224	37	622	920	1,410	99
Rat Walker carcinoma with A-methopterin	186	56	750	905	1,150	107
Gastric carcinoma			296	1,130	536	122
Gastric carcinoma with A-methopterin			268	1,033	480	123
Fibrosarcoma	66	124	218	2,300	252	348
Carcinoma of large intestine	163	32	370	1,070	650	94
Teratoma of testis	120	48	169	1,346	326	187
Spleen from patient bearing gastric carcinoma	206*	24	444	309	950	43
	128†	81				
Regenerating liver	136*	149	2,680	630	4,770	216
	63†	246				
Rat liver (tumor-bearing animal)			1,430	276	2,890	69

* Eluted from Dowex-1 column with 0.1 N HCl.
† Eluted from Dowex-1 column with 0.01 N HCl.

The table also shows the ready incorporation of C^{14} into slices of Walker carcinoma. A considerable difference in degree of incorporation into the uridylic and cytidylic acids may be observed. The ratios of their specific activities rise above normal to from 7 to 10:1. The thymine had an activity 1/25 that of the uracil. In the next experiment, slices from the same tissues used in the previous experiment were incubated with orotic acid plus 1μg. of A-methopterin. No significant effect of A-methopterin on the incorporation of C^{14} into pyrimidines was noted, but the data from the two experiments illustrate the reproducibility of results in the system.

The patterns of incorporation of the orotic acid into the nucleic acid pyrimidines of the human tumors are shown next. The incorporation of C^{14} into cytosine is relatively low in all cases. To one portion of slices of the gastric tumor 100 μg. of A-methopterin was added, with no inhibitory effect. The spleen of the patient bearing the gastric

carcinoma was also studied. The specific activities of the uridylic and cytidylic acids bear somewhat the relationship to one another as in the tumor itself.

Table 1 also shows the results of a similar experiment with regenerating liver, 48 hours after surgery. It will be noted that the ribonucleic acid components show little variation from the normal liver, whereas the activity of the thymidylic acid eluted with 0.1 N HCl is relatively much greater. A small amount of thymidylic acid eluted with 0.01 N HCl gives an even higher specific activity. The nature of the difference between the two types of thymidylic acids is not yet clear, and the low counts make accurate evaluation of the specific activities difficult. It is probable that this result is owing to the synthesis of new nuclear material. The results from a liver of a tumor-bearing rat, though slightly lower, are similar to those of the normal liver.

DISCUSSION

Orotic acid is readily incorporated into the pyrimidines of nucleic acids present in slices of normal tissues, regenerating liver, Walker carcinoma of the rat, and human tumors. The incorporation into the uridylic acid is, in the case of the tumors, of the order 7–10 times as great as that into the cytidylic acid, whereas in normal tissues and regenerating liver the ratio is from 3 to 4:1. (Arvidson and Hammarsten found, using N^{15}-labeled orotic acid, a 7:5 ratio in *in vivo* experiments with rats [1].) In our experiments the reason for the differences in specific activities is not clear. If, in the case of the normal tissues, there were a reservoir of cytidylic acid 3–4 times that of uridylic, and if in tumors there were 10 times the reservoir of cytidylic acid as of uridylic acid, the results could be explained on simple dilution factors alone, assuming an equal rate of synthesis of uracil and cytosine from the orotic acid. In regard to amounts present, a ratio of cytidylic acid to uridylic acid greater than 2 has not been described in most normal tissues. It is possible that the reservoir of cytidylic acid may be somewhat larger than the reservoir of uridylic acid. We cannot be sure of the exact total amounts of the various components present originally, because it is difficult to evaluate the percentage recovery of the various bases. It is interesting to note in the data reported that the amounts of cytidylic acid are roughly twice those of uridylic acid for normal tissues and some of the tumors. Certainly in no case does the ratio approach 10 to 1. It is therefore unlikely that the difference in the specific activities of uridylic and cytidylic acid can be explained by dilution factors alone.

The most common argument used in work with labeled compounds is that the compound with greater specific activity may be the precursor of the compound with the lower specific activity. Using this argument we may say that the uracil group may be the precursor of the cytosine group in our experiment. The argument receives added support in the realization that uracil may be formed from orotic acid by simple decarboxylation. Cytosine may be formed from uracil by the one step of amination, or it may be formed from orotic acid by two steps: first, amination, and then decarboxylation. There is no way of deciding which of the last possibilities is correct, but it is apparent that orotic acid forms the uracil group more readily than it does the cytosine group.

The relatively small amount of DNA precludes any explanation based on dilution factors in regard to the figures on thymine. As noted here, and as will be pointed out in other work on the rates of renewal of RNA and DNA, it is important to refer to specific bases, and it is perhaps misleading to draw general conclusions from the renewal rates of a single component such as the combined phosphorus of all nucleotides. Since the tumor is a rapidly growing system it is difficult to reconcile the large amount of cytidylic acid present with the lower rate of incorporation. The factors of renewal, growth, *de novo* synthesis from nonradioactive precursors, simple exchange mechanisms, and intracellular organization are not easily divorced and evaluated.

The rate of incorporation of radioactivity into the thymine, as compared to the uracil of tumors, is not unlike that found in normal tissue. On the other hand, the rate of incorporation of orotic acid into thymine of regenerating liver is considerably increased over that of nongrowing liver.

Skipper (4) has demonstrated the inhibition of nucleic acid synthesis by A-methopterin as measured by the *in vivo* incorporation of radioactive formate into the nucleic acid purines. The absence of such an effect of A-methopterin in the present work may be owing to lack of permeability or the failure of the single tissue to convert A-methopterin to a more active form. The other possible interpretation of the results is that the rate of renewal of the nucleic acid pyrimidines is independent of the purines and does not involve steps in synthesis dependent upon folic acid.

SUMMARY

The nucleic acid metabolism of rat and human tumors as well as of regenerating liver and normal tissue from the rat has been studied *in vitro* in

slices by observing the incorporation of C^{14}-orotic acid into the pyrimidines of nucleic acid. In most normal tissue the rate of incorporation of the radioactive precursor into the uridylic acid of the nucleic acid was 2–4 times as great as into the cytidylic acid, whereas in all the tumors studied a seven- to ten-fold difference was noted.

ACKNOWLEDGMENTS

I wish to acknowledge particularly the constant help and guidance of Dr. D. Wright Wilson in all this work. I wish to thank Mrs. Natalie G. Aust for her valuable technical assistance. Thanks are due to members of the Department of Surgery for their co-operation in furnishing tissue immediately after its removal from the body, and to the Harrison Department of Research Surgery for preparing the animals with regenerating liver.

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Tumor Production in Rats Injected Intravenously with Oil Emulsions Containing 9,10-Dimethyl-1,2-Benzanthracene*

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Emulsions of fat suitable for intravenous administration have been used in this laboratory as a source of calories, as an aid in the study of lipid metabolism, and as a vehicle for fat-soluble materials. The latter use has been of value in studies with rats on the effects of the intravenous injection of carcinogenic hydrocarbons. Several investigations have been reported in which emulsions or suspensions of carcinogenic compounds have been given intravenously to experimental animals. Andervont and Lorenz (1) reported that mice of the C3H strain developed pulmonary tumors following the intravenous injection of suspensions of 1,2,5,6-dibenzanthracene. Shimkin (6) reported that strain A mice developed pulmonary tumors after intravenous injection of 20-methylcholanthrene in an emulsion. No neoplasms were found by Garay and Berencsi (3) to develop in mice or rabbits after the intravenous administration of suspended 3,4-benzpyrene. The rapid excretion of intravenously administered radioactive dibenzanthracene has been reported by Heidelberger and Jones (5).

The present paper deals with the production of skin tumors in rats following the intravenous injection of emulsions of oil which contained dissolved 9,10-dimethyl-1,2-benzanthracene (DMBA).

EXPERIMENTAL

In preliminary studies the carcinogenic agent was dissolved in corn oil,[1] and the mixture was emulsified by means of a high-speed blender at a 5 per cent concentration of fat and in a nitrogen atmosphere. One per cent each of a soybean phosphatide fraction (4) and a polyglycerol ester[2] were used as emulsifiers and stabilizers, and 5 per cent dextrose was used for tonicity. The particles of fat were approximately 1 μ or less in diameter, and the emulsions were sterilized by autoclaving. The emulsions were injected twice weekly for 3 months into male 150-gm. rats of the Charles River strain (formerly Wistar). 1,2-Benzanthracene, 9,10-dimethyl-1,2-benzanthracene, 20-methylcholanthrene, 1,2,5,6-dibenzanthracene, *p*-dimethylaminoazobenzene, and *m'*-methyl-*p*-dimethylaminoazobenzene[3] were tested in this manner. No tumors were obtained in any animals injected with any emulsion except dimethylbenzanthracene. Survival in all groups, including several in which no injections were given, was poor prior to the fifth month owing chiefly to respiratory disease. The neoplasms which developed in the animals injected with dimethylbenzanthracene were epidermoid carcinomas and papillomas, most of which appeared to arise from the sebaceous glands adjacent to one or both external ears. Infection and hemorrhage at the site of the tumors often caused death, usually after the tumor had reached the approximate size of 2×3 cm.

Several additional groups of rats were tested on the dimethylbenzanthracene emulsion with the same results as before. One of these groups received twice-weekly croton oil applications on several different locations on their backs, but, as with animals in other groups, survival was poor, and the survivors developed tumors only on the head region and not at the site of the croton oil applications.

At this point it became possible to prepare more concentrated fat emulsions by means of high pressure homogenization and to use different stabilizing materials. New experiments were set up using emulsions in which the only carcinogen studied was dimethylbenzanthracene. The emulsions were

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[1] A specially refined corn oil generously supplied by the Corn Products Refining Company.

[2] The Demal-14 was generously supplied by the Emulsol Corporation, Chicago, Ill., and Triton-WR-1339 was generously supplied by the Rohm & Haas Company.

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[3] The authors wish to express their appreciation to Dr. J. A. Miller of McArdle Memorial Laboratory, University of Wisconsin, who made available to them a generous supply of *m'*-methyl-*p*-dimethylaminoazobenzene.

prepared as follows: 42.9 mg. of 9,10-dimethyl-1,2-benzanthracene (Eastman) was dissolved in 15 gm. of warmed corn oil, and 1.0 gm. of a polyglycerol ester of oleic acid (Demal-14) or 0.25 gm. of an aryl-alkyl ether polymer (Triton-WR-1339) was added. This mixture was combined with 82 ml. of 5 per cent dextrose solution which contained 1.0 gm. of cerebrosides,[4] and the entire mixture was homogenized in a high-pressure homogenizer[5] for 20 minutes at a pressure of 3,000 pounds per square inch and a temperature of 88° C. A nitrogen atmosphere was maintained during the homogenization. The size of the fat droplets was determined by visual phase microscopy, and the particles were always 1 μ or less in diameter, with most of them near or beyond the resolving power of the light microscope. The emulsion was collected directly in 150-ml. bottles which were then flooded with nitrogen, tightly sealed, and autoclaved for 15 minutes at 15 pounds per square inch. After cooling, the emulsions were stored at room temperature in a dark place until used. The emulsions contained 0.429 mg. of dimethylbenzanthracene per milliliter.

Female rats of the Sprague-Dawley strain[6] weighing about 150 gm. were used in all the following experiments except one in which weanling rats of the same strain and sex were used. The rats were given approximately twelve weekly injections of either the Demal- or Triton-stabilized emulsion. All injections were given through the veins of the tail while the animal was lightly anesthetized with ether. Several groups of animals received a comparable number of injections of an emulsion containing Demal or Triton but no DMBA. Several other groups of animals served as uninjected controls. All the animals were maintained on stock ration[7] and housed in group cages. In Table 1 are presented the data pertinent to the experiment and the results in terms of the number of animals which developed tumors and the total number of tumors observed.

TABLE 1

INCIDENCE OF TUMORS IN RATS FOLLOWING THE INTRAVENOUS INJECTION OF EMULSIONS CONTAINING 9,10-DIMETHYL-1,2-BENZANTHRACENE (DMBA)

Group no.	Emulsion no.*	No. of weekly injections	Total DMBA given (mg.)	No. rats	Survivors at 19 wks.	Animals with tumor(s) at 19 wks.†	25 wks.	32 wks.	Total tumors at 32 wks.
38I	B	13	6.4 (5.7–7.7)	30	23	7	15	17	24
38II	B	12	5.5 (4.8–6.2)	30	16	3	5	12	23
39I	A	12	5.5 (5.1–6.2)	30	20	1	1	5	8
39II	A	10	4.1 (3.7–4.8)	22	11	0	4	7	14
40A‡	A	13	3.7 (3.6–3.9)	20	8	3	5	5	8
40B‡	A	13	3.3 (3.0–3.5)	20	16	4	6	6	9
TOTAL				152	94	18	36	52	86

* Emulsion No. A contained 15 per cent corn oil, 1 per cent cerebrosides, 1 per cent Demal-14, 0.0429 per cent 9,10-dimethyl-1,2-benzanthracene, and 4.3 per cent dextrose.
Emulsion No. B was similar to No. A, except that the 1 per cent Demal-14 was replaced with 0.25 per cent Triton.

† No tumors were observed prior to the nineteenth week after the first injection of the emulsion.

‡ Weanling rats.

For histological purposes, some biopsies were taken of various tumors, and at necropsy sections of all tumors and of many normal-appearing tissues were obtained. The tissue was fixed in Zenker's fluid or 4 per cent formaldehyde solution, and the mounted sections were stained with hematoxylin and eosin, Masson's trichrome, phosphotungstic acid hematoxylin, or Sudan IV. A listing of location and kinds of tumors produced is given in Table 2. Figures 1, 2, and 3 are photomicrographs of some of these tumors.

RESULTS AND DISCUSSION

A fairly high incidence of skin tumors in rats resulted when 9,10-dimethyl-1,2-benzanthracene was administered intravenously in the form of emulsions. As shown by the data in Table 1, approximately 33 per cent of all the animals injected with these emulsions developed one or more tumors adjacent to the ears or on the ventral trunk.

[4] A purified cerebroside preparation kindly supplied by Dr. R. J. Vander Wal of the Armour Company, Chicago, Ill.

[5] Model Junior 50 Viscolizer, Cherry-Burrell Company, Chicago, Ill.

[6] Obtained from the Charles River Breeding Laboratories, Boston, Mass.

[7] Gaines Krunchon, Gaines Division, General Foods, Kankakee, Ill.

When based on the number of surviving animals at the time the first tumor was recognized, the percentage of rats with tumors was approximately 55 per cent. Death in most cases was caused by a respiratory infection which also was responsible for the fatalities in the control groups that received no injections of emulsion. The majority of fatalities occurred prior to the fifteenth week. Within the range of total amount of carcinogen given in these experiments, little can be said regarding correlation between dose and response. In several additional experiments, weanling female rats that received a total of 1 mg. of dimethylbenzanthracene in six injections had a tumor incidence of 10 per cent in 32 weeks. No change in kind or incidence of tumors resulted from substituting the polyglycerol ester (Demal-14) for the aryl-alkyl ether (Triton WR-1339) as part of the stabilizing system for the emulsion. In all these experiments cerebroside made up the rest of the stabilizing system. In the preliminary experiments in which the remainder of the stabilizing system was phosphatide instead of cerebroside, a low incidence of tumors was obtained with this carcinogenic agent, and most of these arose in the region of the ears. Other normally potent agents such as methylcholanthrene were without effect when contained in the phosphatide-Demal stabilized emulsions. This difference between the phosphatide- and cerebroside-stabilized emulsions may be due (*a*) to an influence of these substances on tumor formation, (*b*) to an effect of these materials on the carcinogenic agents prior to injection (e.g., the phosphatides might participate in a coupled oxidation with the carcinogen), (*c*) to an influence which these substances might have on the extent and speed of emulsion removal by the skin or sebaceous glands, or (*d*) to a difference in the strain and sex of rat used in the two different sets of experiments. Studies are now in progress to elucidate which, if any, of these possibilities is responsible. The carcinogens which were tested with the phosphatide-stabilized emulsions are now being retested in cerebroside-stabilized emulsions.

TABLE 2

DESCRIPTION OF TYPICAL TUMORS PRODUCED IN RATS FOLLOWING THE INTRAVENOUS INJECTION OF EMULSIONS CONTAINING 9,10-DIMETHYL-1,2-BENZANTHRACENE

Tissue of origin	Histological characterization of tumor	No. of tumors
Mammary gland, left side	Adenocarcinoma	8
	Adenoacanthoma	2
	Adenoma	1
	Fibroadenoma	1
Mammary gland, right side	Adenocarcinoma	11
	Adenoacanthoma	2
	Adenoma	1
Sebaceous gland, left ear duct	Adenoma	1
	Epidermoid carcinoma	5
Sebaceous gland, right ear duct	Epidermoid carcinoma	6
Subcutaneous tissue, left groin	Fibroma	1
Liver, spleen, nodes	Monocytic leukemic infiltrate	2

The tumors in the preliminary groups in which phosphatide was present in the emulsion represented keratinizing squamous-cell tumors of sebaceous gland type, arising adjacent to the external auditory canal. These tumors were similar to those induced by oral 2-acetylaminofluorene (2) and by oral administration of benzidine or by subcutaneous injection of benzidine in unemulsified olive oil (8). Twelve per cent of 88 animals injected with dimethylbenzanthracene in phosphatide-stabilized emulsions developed these tumors, one of which was bilateral. Forming beneath the skin just anterior and inferior to the external ear, the tumors grew to large sizes, extending over the necks of the animals internal to the mandible, and along the base of the skull. When small, the tumors were coarsely lobulated and semi-firm. All which were of moderate or large size showed puriform liquefaction, and most of them contained masses of gray-white cheesy material. Histologically, the tumors were cystic, papillary lesions with bands of keratinized squamous epithelium, clublike rete pegs, and masses of sebaceous cells. There were groups of atypical prickle and basal cells with nuclear and cytoplasmic variation, many mitoses, and stromal invasion. The cystic spaces were filled with lipid-rich sebum or keratin. These tumors were invasive and histologically malignant, and two tumors had metastasized to the lung.

Ten per cent of 177 rats which received dimethylbenzanthracene in cerebroside-stabilized emulsions also developed keratinizing squamous-cell tumors of sebaceous gland type (Fig. 1). Thirty-five per cent of these were bilateral. This can be compared with the 9 per cent incidence of bilaterality in the preliminary experiments and the 10.6 per cent incidence in the report of Spitz *et al.* (8). In none of these animals were metastases demonstrated. Thirty-two per cent of these rats also developed numerous tumors over the course of the "mammary line" on the ventral surface of the trunk. Six per cent of animals developed both types of tumors, and 57 per cent of animals with "ear" tumors also presented mammary tumors. Some of the mammary tumors were bilateral, and a number were multiple. The tumors arose in the corium and spread in the subcutaneous tissue, growing to a size of more than 6 cm. and to a weight of more than 50 gm. Most of those which

were more than 1 cm. in diameter had invaded underlying muscle, and many were ulcerated. No distant or lymph node metastases were found. It is probable that some of the skin tumors were metastatic satellites, e.g., the flank tumor which was on the same horizontal level with the tumor over the left lower abdomen in rat 38II-3, described below.

Some tumors produced by cerebroside-stabilized emulsions grew into massive neoplasms. One animal (38II-3) developed six large tumors: (*a*) 3×2×1.5 cm. in the right clavicular region, (*b*) 5×3×2 cm. over the right chest wall, (*c*) 7×4×2 cm. over the right inferior costal border and abdomen, (*d*) 9×6×4 cm. over the entire left chest and upper abdomen, (*e*) 2.5×1.5×1.0 cm. over the left flank, and (*f*) 1×0.8×0.6 cm. over the left abdomen. This represented a total of 148 gm. of tumor in a 368-gm. rat.

Although there was relatively great variation in histologic picture, two major types of tumor were produced by the emulsions containing cerebroside (Table 2). One was of epidermoid and sebaceous gland type, the other of apparent mammary gland derivation. It should be noted that both these types represented tumors of skin and dermal adnexae. The variations represented transition from one type of breast tumor to another, differences in apparent malignancy, and variations in stromal activity. Two animals with leukemic infiltrates in liver, spleen, and lymph nodes were also identified. On microscopic examination atypical acini and small cysts, sheets, and clumps of cells were found in a generally active, fibroblastic stroma. Many of the acini were thyroid-like in size and contained colloid-like material which did not include lipid. There were many mitoses, and there was invasion of stroma and adjacent muscle. Some of the tumors exhibited apparent transition from fibroadenoma to adenoma to adenocarcinoma (Fig. 4). In some there were squamous-lined ducts and sheets of prickle cells.

The animals in the preliminary experiments died within a few weeks of the development of tumors. Necrosis and fatty change of spleen and liver, pneumonia, and other infections were found at necropsy. Rats that received a cerebroside-stabilized emulsion, however, lived for months after development of tumors of both types, and some lived for weeks after ulceration of these tumors. Some were finally killed. A few appeared to die of "cancer cachexia." Some died of respiratory or generalized infection.

The cutaneous localization of these tumors, induced by a parenteral agent in a stable emulsion is striking. Simpson and Cramer (7) demonstrated that, after topical administration, 20-methylcholanthrene is concentrated in sebaceous glands. Suntzeff *et al.* (9) showed that the development of epidermal carcinomas depends upon the presence of dermal adnexae. It remains to be determined whether the activity of intravenously administered dimethylbenzanthracene is concentrated in sebaceous glands, whether the carcinogen is modified or activated in sebaceous glands, or whether there is a special susceptibility of dermal organs to the carcinogenic effect of these fat-soluble agents administered in stable emulsion.

It is obvious that the studies reported here are incomplete. Such fundamental aspects as the responses of tumor-susceptible strains of animals versus nonsusceptible strains remain to be studied. However, the experiments reported in this paper demonstrate clearly the practicability of using properly prepared fat emulsions as vehicles for the intravenous administration of fat-soluble carcinogenic compounds. The results of these experiments demonstrate the desirability of the use of such emulsions in the study of tumor production; and, conceivably, fat emulsions also offer a practical route of administration of compounds of possible chemotherapeutic value in the field of tumors.

SUMMARY

The intravenous administration of fat emulsions which contained 9,10-dimethyl-1,2-benzanthracene caused a high incidence of skin tumors in female rats of the Sprague-Dawley strain. Fifty-five per cent of the surviving animals developed keratinizing squamous-cell tumors of the sebaceous gland type and/or adenocarcinomas which were usually located over the course of the "mammary line" on the ventral surface of the trunk. Only two animals developed neoplasms which were not of skin or dermal adnexae derivative.

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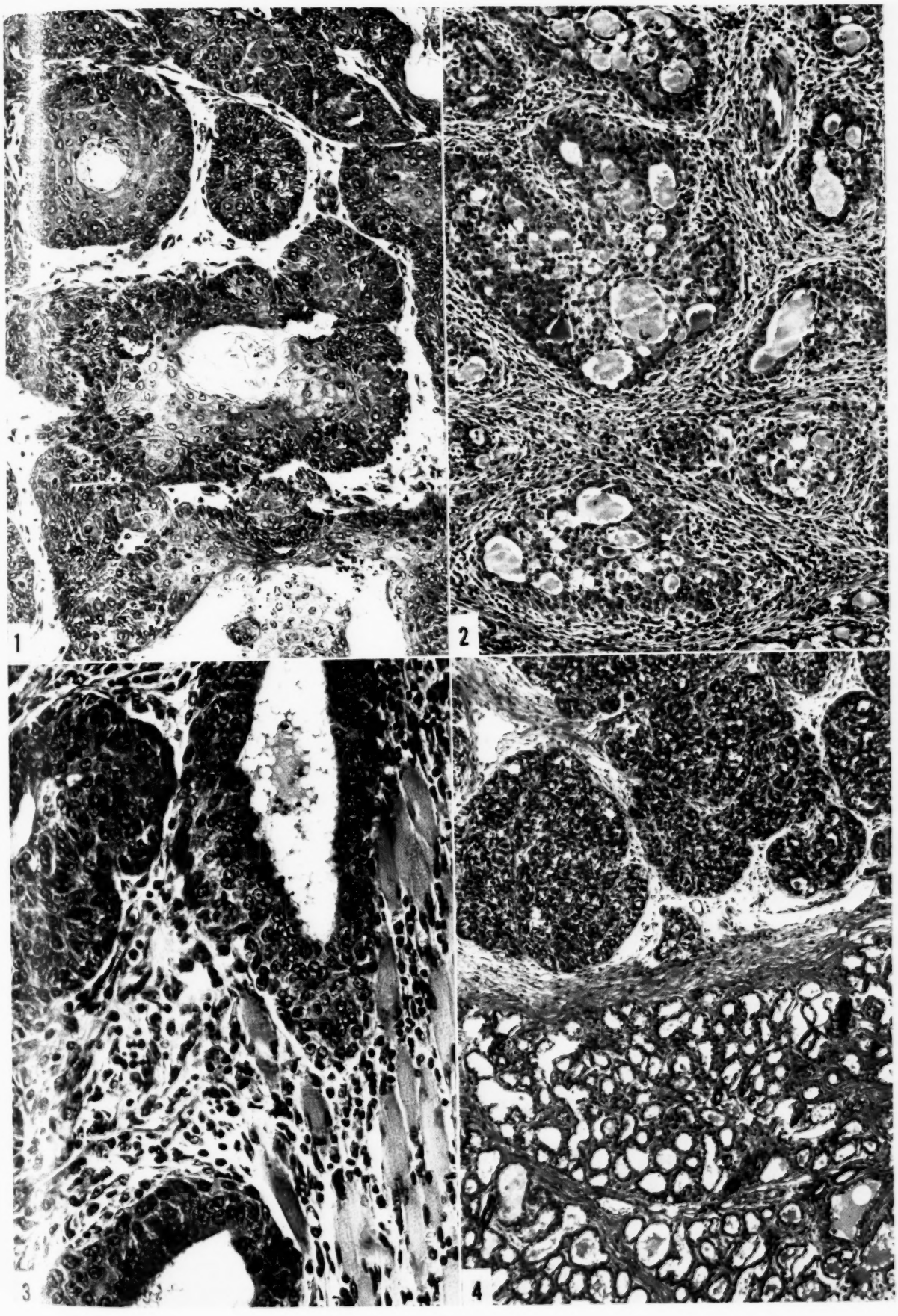
Fig. 1.—Epidermoid carcinoma arising in the sebaceous gland adjacent to the external ear. Rat 39II, No. 15; dimethylbenzanthracene, cerebroside stabilizer, and Triton.

Fig. 2.—Secretory type adenocarcinoma, invasive, low grade of malignancy, of right pectoral region. Rat 38I, No. 22; dimethylbenzanthracene with cerebroside stabilizer and Triton.

Fig. 3.—Adenocarcinoma of right breast. Rat 38I, No. 12; dimethylbenzanthracene, cerebroside stabilizer, and Triton. Note invasion of skeletal muscle.

Fig. 4.—Adenoma of secretory type arising adjacent to atypical adenomatous hyperplasia of breast. Rat 38II, No. 3; dimethylbenzanthracene, cerebroside stabilizer, and Triton.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Book Reviews

A Symposium on Steroid Hormones. EDGAR S. GORDON (ed.). Madison: The University of Wisconsin Press, 1950. Pp. 396. $6.50.

This book is a collection of the major papers and round table discussions which were presented at the symposium on steroid hormones held in Madison in September, 1948; it will be of interest to anyone doing research on steroid hormones. Many of the papers have been revised since the symposium was held, and some contain references to research published as late as 1950. Most of the papers deal almost exclusively with research done by the authors, but they contain sufficient references to indicate their relationship with other work on steroid hormones. Each of the authors is well recognized in his field and in most cases has presented a summary of research which extended over several years.

The book begins with historical reviews of the isolation and the chemistry of the steroids and includes a round-table discussion of the relation of structure to biological function of steroids. It includes papers on the biosynthesis, metabolism, and excretion of steroid metabolites, as well as a discussion of the relationships between the pituitary and the target organs which produce steroid hormones. Topics related to the metabolic effects of the steroid hormones include: the mechanism of the protein anabolic action of testosterone propionate; metabolic effects of adrenal steroids; factors affecting endometrial growth in monkeys; the influence of hormones on behavior; and the role of hormones in sex differentiation. Papers concerned with the clinical aspects of steroid hormones include discussions of the metabolism of convalescence and the relation of steroid hormones to cancer.

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Symposium on Radiation Genetics. Journal of Cellular and Comparative Physiology, Vol. 35, Suppl. 1, 1950. Pp. 210.

This volume contains all but two of the papers given at the first "Information Meeting for Biology and Medicine" sponsored by the Biology Division of the Oak Ridge National Laboratory and held in March, 1948. Perhaps the greatest failing of the publication is that it did not appear for 2 years. However, some of the articles have been brought more up to date through later addenda.

Almost a third of the volume is devoted to a brilliant discussion of some particularly important problems in radiation genetics by H. J. Muller. He emphasizes that people who use radiations should be aware of the fact that linearity of the dosage-mutation curve extends to the lowest doses—that there is no threshold for the genetic effects. Paradoxically, the relationship is too nearly linear at higher dosages, probably due to mutual cancellation of opposing factors, which individually would cause departure from linearity. There is a discussion of the target theory and of chromosome breakage and attachment. Professor Muller points out the great need for more investigations carried out directly on mammals if the possible effects of radiation on the human population are to be assessed. Finally, there is an extensive discussion of the possibility that the major portion of general somatic or "physiological" effects of x-rays are due to chromosome breakages.

In the next article Karl Sax gives a lucid, nontechnical account of the types of chromosome breaks induced by x-radiation in pollen grains and the dosage-effect relationship. Subsequent papers are devoted to the different relationship obtained when fast neutrons are used (N. H. Giles and Alan Conger) and the effects of radiation on mitosis in grasshopper neuroblast cells grown in tissue culture (J. Gordon Carlson). Some of the genetic results induced by exposing corn seeds during the Bikini test are reported by L. F. Randolph. Except for a puzzling difference in the frequency of chlorophyll-deficient sectors, the results were approximately equivalent to those produced by a 15,000 γ x-ray dose.

Four of the papers deal with radiation-genetic effects on microörganisms. Perhaps the most pertinent was contributed by R. F. Kimball, on the use of Paramecium in radiation genetic research. Owing to the experimental plasticity of the life cycle, it is relatively simple to isolate mutational from other effects of radiations. He finds that lethal and semi-lethal mutations account for most of the deleterious effects. The group from the University of Texas (O. Wyss, F. Haas, J. B. Clark, and W. S. Stone) briefly summarize their work (to 1948) on ultraviolet effects on bacteria mediated by treated broth. This paper suffers particularly from the delayed publication, and more current and complete accounts are available elsewhere. Other papers deal with the application of radiation to produce mutations in fungi (E. L. Tatum) and bacteria (H. H. Plough) and have a limited bearing on the main themes of the conference, insofar as they are concerned primarily with the mutant characters rather than the mutation process. D. Mazia and G. Blumenthal report that pepsin incorporated in a thin film with a substrate protein is unusually sensitive to x-radiation, 100 r having distinct effects.

In the final article Sewall Wright shows the equations for the changes in a population subjected to radiation or other circumstances leading to an increased mutation rate. Both Muller and Wright emphasize the importance of a slight amount of heterozygous effect in detrimental recessive mutants. Because of the much greater frequency of heterozygotes, any character manifest in this condition becomes much more important than if it were

completely recessive—and, incidentally, the theory is simplified. Wright's final paragraph deserves direct quotation: "The principal conclusions of this analysis are first that there are such enormous gaps in our knowledge that no judgements of the genetic consequences of radiation in man can be taken very seriously. There is, however, a strong possibility that cumulative doses of the order of 300 r may have important effects on the offspring and descendants of those affected, and doses as small as 30 r may not be negligible. On the other hand there is little or no threat to the persistence of a population as a whole from this cause."

In spite of its tardiness, the symposium is a useful review of the genetic aspects of radiation research. The terminology and argument are addressed primarily to the geneticist, but the problems raised bear on many areas of biological research as well as on the social implications of the increased use of radiations and radioactive products.

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Beiträge zur Anwendung der Isotopentechnik in Biologie, Klinik und Therapie. In French and German. Published by the Commission on Isotopes of the Swiss Academy of Medical Sciences. Basel: Benno Schwabe & Co. Imported by Grune & Stratton, Inc., N.Y. Pp. 213. 15 fr.

The monograph, a form of presentation of scientific information in use in German-speaking countries for many years, has only lately been adopted by English-speaking scientists. It serves as a kind of interim report or review of work in progress, and lays no claim to permanence or completeness. Especially when it consists of articles by several authors, each a specialist in his field, the monograph is uniquely useful for introducing new subjects to a wide scientific public. The present volume contains review articles on the use of isotopes in biological research and medical therapy. It is expressedly not a text or a handbook but rather a guide and introduction to the subject for the biologist and physician. Even though the short articles cannot survey more than a fraction of each subject, the reader will find the appended well chosen bibliographies amply sufficient as an introduction to a field.

The first part of the book deals with selected topics in isotope research, mainly biochemical, and aims at introducing this important new tool to the biochemist. The second part is concerned with clinical aspects and therapeutic use of isotopes, and would be of importance to the physician, especially in the cancer field.

Since most of the work on isotopes has been done in this country, and many books and reviews on the subject already exist here, this volume would be of use mainly to German- and French-speaking scientists, although the article by Vannotti, Closuit, and Jaccottet (in clear and easy French) on the use of radioactive iron in distribution studies in normal and abnormal animals, besides giving a comprehensive and excellent review of past work, describes the very thorough research and conclusions of the authors themselves. Moreover, the article by Joyet (in French) on the theory of dosage calculations in application of radioactive isotopes is certain to be of use to the physician interested in isotope administration. The article by Müller (in German) also gives a review of the author's own very ingenious methods and practical experiences in the administration of solutions and suspensions of radioisotopes to cancer patients. He describes, for instance, the use of coarse suspensions of radioactive zinc sulfide in the treatment of lung cancer. The radioactive particles are caught in the lung capillaries and can thus be localized in that organ.

The volume also contains a very short introduction (in German) to the physics of isotopes. Furthermore, a review by Bernhard (in German) on research in metabolism with the use of isotopes, which describes studies in fat metabolism, a field to which the author has himself contributed; sterol and carbohydrate metabolism, briefly; amino acid, purine and blood pigment metabolism, more thoroughly. The list of textbooks and publications appended to this article is exceptionally comprehensive. There follows a very detailed description by Joyet (in French) of working methods for the use of radioactive elements, mainly potassium and calcium, including descriptions of measuring devices, correction tables, and working outlines from injection of the active element to combustion of tissues and determination of their "selectivity" for the radioactive element. There is also an article by Bernhard (in German) on the use of heavy hydrogen in biochemistry, mainly in connection with fat metabolism; and a short review of safety measures recommended for radiation laboratories and clinics. The book ends with a list of radioisotopes available through the U.S. Atomic Energy Commission.

It is disappointing to find no mention made of the technic for the use of radioactive carbon, surely the most important of the isotopes in biological research, nor a discussion of the excellent recent advances in carbohydrate metabolism with the aid of carbon-14.

As a whole, the book is to be warmly recommended to physicians and biologists who wish to enter the field of isotope studies, mainly as a basis for further reading in the subject.

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Annual Reports on the Results of Radiotherapy in Cancer of the Uterine Cervix. Vol. 6. By DR. J. HEYMAN (ed.). Stockholm: P. A. Norstedt & Sons. Pp. 172.

Primary Carcinoma of the Liver. By CHARLES BERMAN. London: H. K. Lewis & Co. Ltd. Pp. 164. 35 s.

Die Frühdiagnose des Uterusca cinoms. By HANS LIMBURG. Stuttgart: Georg Thieme Verlag. Pp. 158. $8.50.